Separation of Megakaryocytes From Mouse Bone Marrow by Velocity Sedimentation

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A technique for separating mouse bone marrow megakaryocytes by unit gravity velocity sedimentation is described using a modified sedimentation chamber. The velocity sedimentation profile for megakaryocytes comprises three peaks corresponding to sedimentation velocities of 30, 60, and 100 mm/hr. Morphologic examination of megakaryocytes in the 30 mm/hr peak reveals them to be primarily immature with the latter two peaks corresponding to larger, more mature megakaryocytes.

The stem cell-megakaryocyte-platelet system represents a unique differentiating system in which hormonal and probably cell-mediated controls act to regulate the number and function of each cell compartment. In the case of the small fraction of stem cells in normal bone marrow, investigators interested in studying this population of cells have had to design techniques for their separation and subsequent concentration. Similarly, since megakaryocytes also constitute a small proportion of the total nucleated cells (approximately 0.05%), a method for the separation and concentration of these cells would aid in analyzing quantitatively their functions as well as the kinetics of their production.

The unit gravity velocity sedimentation technique has been used to separate various mammalian cell populations on the basis of differences in cell volume. Since megakaryocytes are among the largest cells in bone marrow, this technique should afford a convenient means for their separation. In this study, the separation of megakaryocytes by a modified velocity sedimentation technique is described.

MATERIALS AND METHODS

Random-bred, male ND2 mice (20–25 g) were killed by chloroform overdose, and both femurs were removed and cleaned of excess tissue. In order to optimize the yield of megakaryocytes, which are rather fragile to handling, the following technique was used: Only the proximal end of each femur was cut and a 25-gauge needle attached to a 3-ml syringe inserted therein. The needle was bent at a right angle to facilitate backflushing the marrow contents with 2 ml of phosphate-buffered saline (PBS) into a siliconized flask. The cell suspension was rendered monodispersed by gentle pipeting, and stromal fragments were removed by filtering the suspension through four layers of nylon gauze. Little or no loss of megakaryocytes resulted from this latter procedure.

The procedure of Miller and Phillips was altered such that the cell suspension was brought...
gently upon a preformed gradient of 0.5%, 2% (w/v) bovine albumin (Fraction V, Sigma Chemical, St. Louis, Mo.) in PBS in a modified chamber illustrated in Fig. 1. This arrangement permitted measurement of sedimentation rates of megakaryocytes which otherwise began sedimenting during gradient formation. In one experiment, a cell suspension containing 5 \times 10^7 nucleated bone marrow cells with 10.8 \times 10^5 megakaryocytes in a total volume of 10 ml of PBS was applied to the gradient, and in two subsequent runs, 2.2 \times 10^7 nucleated cells containing 7.8 \times 10^5 megakaryocytes in 10 ml of PBS were used. After reaching the starting position (arrow, Fig. 1), the cells were allowed to sediment for 30-45 min at 4°C. Ten-milliliter fractions were then collected, and the first two fractions were discarded. Subsequent fractions were collected continuously at a flow rate of 20 ml/min until the first vestiges of the clearly discernable starting layer of cells appeared in the fractions. At this point, the collection was stopped, and the remainder of the gradient (empirically found to contain more than 90% of the nucleated cells initially placed on the gradient) was discarded. Thus, all of the megakaryocyte-rich fractions together comprised only about 4%–8% of the total nucleated cell count. The total nucleated cell count in the initial cell sample placed on the gradient was determined by means of an electronic particle counter (Coulter Counter Model B) using a 0.01-ml sample suspended in 10 ml of PBS to which three drops of Zaponin (Coulter Diagnostics, Hialeah, Fla.) was added. Cell concentrates were prepared from each fraction by centrifugation at 175 g for 5 min and resuspension of the cell pellet in approximately 0.2 ml PBS. The total nucleated cell count in this pellet was determined electronically using a 0.2-ml sample suspended in 5 ml of PBS to which three drops of Zaponin was added. In addition, the number of megakaryocytes in the initial cell sample placed on the gradient was quantitatively determined following its sedimentation on glass slides as follows: A 0.02-ml aliquot was placed in the center of a well (absorbent filter Schleicher and Schuell No. 602 H) in an apparatus described by Sayk, to which had been added four drops of 10% fetal calf serum in PBS. Similarly, the number of megakaryocytes in each fraction was determined. After air drying for approximately 20 min, the preparations were fixed in absolute methanol for 3 min and then stained by a modified May-Grünewald Giemsa technique.

The megakaryocytes in each stained preparation were counted microscopically and, knowing the volume of the cell sample placed in the apparatus and the total volume of cell sample from which the aliquot was withdrawn, the total number of megakaryocytes per fraction was expressed both as a percentage of the total number of megakaryocytes placed initially on the gradient and as a percentage of the total nucleated cells in the same fraction. The average sedimentation velocity was measured as described previously, assuming that each millimeter of length in the cylindrical portion of the gradient contained 5.03 ml of fluid.
RESULTS AND DISCUSSION

The composite velocity sedimentation profile of mouse bone marrow megakaryocytes obtained from three experiments is shown in Fig. 2. The number of megakaryocytes recovered from each of the three gradients expressed as a percentage of the total number initially placed on the gradients was 66%, 71%, and 97%. Three peaks were obtained which were characterized by an average sedimentation velocity of approximately 30, 60, and 100 mm/hr. Since the sedimentation rate in millimeters per hour is approximately equal to $R^2/4$, where $R$ is the radius of the cell being sedimented in microns, the volume of megakaryocytes in these fractions could be determined using the derived value of $R$ and assuming the megakaryocytes in solution to be spherical. These rates corresponded to megakaryocyte volumes of approximately 7000, 14,000, and 32,000 cu µ, respectively. By virtue of their larger cell volume, megakaryocytes in the last two peaks would correspond to those that were the more mature, as has been described earlier. This was confirmed cytologically by their more abundant, granular, and lighter staining cytoplasm (particularly by cells in the last peak) (Fig. 3), as compared to megakaryocytes in the smaller cell volume peak. The latter comprised a younger, maturing population with less cytoplasm that was more intensely basophilic (Fig. 4).

The degree of separation of megakaryocytes from the other cells in the marrow suspension was such that megakaryocytes were enriched from 0.03%, to as high as 1% of cells in certain of the megakaryocyte-rich fractions. The remaining cells comprised cell clumps which, when exposed to the cytoplasmolytic action of Zaponin in the process of electronic cell counting, yielded the observed nonmegakaryocytic component of the cells in each cell fraction. By using larger sedimentation chambers to increase the number of bone marrow cells that can be separated efficiently, one could potentially obtain fractions

![Fig. 2. The sedimentation velocity profile of mouse bone marrow megakaryocytes (average of three experiments) expressed as (1) the percent of total nucleated cells per fraction (0-0) and (2) the percent of total megakaryocytes (8-8) initially placed on the gradient. This profile represents less than 10% of the total nucleated cells initially placed on the gradient, the remainder not being shown here.](image-url)
sufficiently enriched in megakaryocytes and containing sufficiently high numbers of these cells to permit counting and direct cell volume determinations using an electronic particle counter. Concomitant physical separation of megakaryocytes by velocity sedimentation into possible functionally discrete populations will be used to analyze the kinetics of megakaryocyte production in vivo and the manner in which control mechanisms govern this process.

REFERENCES

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