Application of Flow Cytometry and Cell Sorting to Megakaryocytopoiesis

By A. Nakeff, F. Valeriote, J. W. Gray, and R. J. Grabske

We have employed flow cytometry (FCM) and cell sorting to quantitate and study megakaryocytes in mouse and rat femoral marrow following their 20- to 30-fold concentration by centrifugal elutriation (CE). This enrichment of megakaryocytes permitted the first determination of their DNA-related fluorescence by FCM analysis following DNA staining. Fluorescence distributions of CE-enriched cell fractions following supravital staining with Hoechst 33342 were similar to those following chromomycin A3 staining of ethanol-fixed cells. Microscopic examination of cells sorted onto glass slides on the basis of their DNA-related fluorescence following supravital staining together with specific acetylcholinesterase staining for megakaryocytes indicated that megakaryocytes generally increased in cell size with increasing DNA content. This technologic application represents a significant advance in the study of megakaryocytopoiesis, since the kinetics of either the normal or perturbed population can now be studied rapidly and quantitatively.

RAPID ADVANCES in the study of the cell kinetics of megakaryocytes have been limited by two problems. The first has been the difficulty of obtaining sufficient quantities of these cells for adequate study; their frequency in normal mouse marrow is about \( 5 \times 10^{-4} \), and this must be substantially greater for effective study with present-day instrumentation.\(^1\) Although megakaryocytes have been concentrated 10- to 15-fold by velocity sedimentation\(^1\) and discontinuous albumin density-gradient centrifugation\(^2\) and about 100-fold by a modified combination,\(^3\) these techniques require a substantial amount of time and involve extensive manipulation of the megakaryocytes. The second problem has been the lack of suitable techniques for rapidly and quantitatively analyzing their DNA. Quantitative determination of megakaryocyte DNA has been accomplished mainly by scanning microspectrophotometry of single megakaryocytes on slides or in tissue sections, generally following Feulgen staining.\(^6\) This type of analysis is extremely time-consuming.

This report presents new approaches to the measurement of megakaryocyte DNA that effectively circumvent the limitations described. Large numbers of rodent bone marrow megakaryocytes are rapidly concentrated by centrifugal...
elutriation (CE), and the distribution of chromomycin A3 or Hoechst 33342 fluorescence among the concentrated cells is quantitatively determined by passage through a flow cytometer.

**MATERIALS AND METHODS**

**Preparation of Bone Marrow (BM) Cell Suspension**

Mice were killed by cervical dislocation and rats by ether asphyxiation, and their femurs were removed. To optimize the yield of undamaged megakaryocytes, we cut the proximal end of each femur and backflushed the contents with 1.5 ml (mouse) or 10 ml (rat) of 0.5% bovine serum albumin (BSA) (fraction V, Sigma) in phosphate-buffered saline (PBS). BM cells were monodispersed by gently passing them twice through a 25-gauge needle, and the entire suspension was adjusted to approximately $5 \times 10^6$ cells/ml with 0.5% BSA in PBS.

**Megakaryocyte Quantitation**

In order to examine and count megakaryocytes in the starting cell suspension and in the fractions obtained following CE, samples were sedimented on glass slides using a Sayk apparatus as described previously. Briefly, a 50-μl sample of 2% BSA in PBS was placed in each chamber, and 15 min later a 25-μl sample of marrow was placed in the center of the chamber and sedimented for 15 min. After complete drying in air for an additional 30 min, the slides were fixed in 5% glutaraldehyde (Electron Microscopy Sciences, Fort Washington, Pa.) for 10 min and washed for 1 min in 0.1-M sodium phosphate buffer (pH 6). The slides were then stained for 3 hr at room temperature for megakaryocytic acetylcholinesterase (AChE) activity as described previously and stained with hematoxylin. The number of megakaryocytes in each preparation was determined using light microscopy at a magnification of 125. A total nucleated cell count was obtained using either a counting chamber and 0.5% methylene blue or an electronic cell counter (Celloscope, Chicago, Ill.) with cetrimide as a cytoplasmolytic agent.

**CE**

CE separates cells of different sizes by taking advantage of their differences in sedimentation velocity. The sedimentation velocity of a cell is determined, according to Stokes' law, primarily by its radius and to a lesser extent by its density. The use of the Beckman elutriator for the enrichment of specific cell types from a complex mixture of cells has been described in detail elsewhere. The mix of cells to be separated is placed into a specially constructed chamber mounted in the elutriator centrifuge rotor. A flow of buffer is pumped (by way of rotating seals) into the peripheral end of the chamber, and it leaves the chamber at the central end. The chamber is narrow at the peripheral end and gradually widens toward the central end. Thus the movement of buffer is relatively fast where it enters, and it becomes progressively slower as it moves into the wider parts of the chamber. The cells in the chamber are acted on by two opposing forces, the centrifugal force generated by the rotation of the chamber and by the flow of buffer moving through the chamber. During a run the cells move to the portion of the chamber where the sedimentation velocities of the cells equal the flow rate of the buffer. The larger cells accumulate at the peripheral end of the chamber, and the smaller cells move toward the central end. After the positions of the cells stabilize in the chamber, the smaller cells can be selectively collected either by slightly increasing the flow rate of the buffer or by reducing the centrifuge speed. Both of these changes allow all of the cells in the chamber to move slightly in the central direction. Any cell whose sedimentation velocity is less than the minimum rate of buffer flow is swept out of the chamber and collected.

The elutriation buffer was PBS supplemented with 0.5% BSA. The buffer was precooled to 4°C before beginning the separation and maintained at that temperature for the duration of the experiment. The megakaryocytes are the largest type of cell in the BM, and they can be enriched by using a single combination of buffer flow rate (25 ml/min) and centrifuge speed (2200 rpm or 465 g). Under these conditions all cells with a sedimentation velocity of 10 mm/hr (corrected to unit gravity) passed through the centrifuge, and all cells of greater sedimentation velocity were retained in the separation chamber.

BM cells were introduced into the elutriator at $5 \times 10^6$ cells/ml in 40 ml or $1 \times 10^6$ cells/ml in 200 ml of 0.5% BSA in PBS. After loading the initial cell suspension, between 200 and 600 ml of cell-free medium was used to clear the cells sedimenting at less than 10 mm/hr from the rotor. The rotor was
then allowed to come to a stop, and the cells sedimenting at more than 10 mm/hr were collected as they flowed out of the system. Whenever a small pellet of cells accumulated at the bottom of the separation chamber during the runs, the pelleted cells were recovered by resuspending the pellet using a 5-ml syringe fitted with an 18-gauge needle inserted through the exit hole in the separation chamber. These were then added to the fraction sedimenting at more than 10 mm/hr.

Staining of Megakaryocyte DNA for FCM Analysis

Cells that had been separated by CE were centrifuged at 200 g for 10 min. The supernatant was withdrawn by vacuum, and the cell pellet was resuspended by gentle pipetting in 5 ml of cold 70% ethanol. After fixing for 30 min, the cells were centrifuged at 700 g for 10 min; the supernatant was decanted, and the cell pellet was resuspended by pipetting in 3 ml of cold chromomycin A3 staining solution: 10 mg chromomycin A3 (Calbiochem), 1.5 g MgCl$_2$·$6$H$_2$O, and 500 ml H$_2$O. Chromomycin A3 is presumed to be relatively DNA-specific and to bind preferentially to guanine-cytosine-rich DNA.$^{20}$ After staining for 30 min at room temperature, the suspension was introduced into the flow cytometer.

Cells were supravitally stained with the dye Hoechst 33342 (kindly supplied by Dr. Loewe, Hoechst AG, Frankfurt, West Germany). Hoechst 33342 is an analog of Hoechst 33258 that is presumed to be mostly DNA-specific with a preference for adenine-thymine-rich DNA.$^{21}$ In this procedure, approximately 5 $\times$ 10$^6$ cells in 5 ml PBS were added to an equal volume of 20-μM Hoechst 33342 in PBS and incubated at 37°C for 2 hr. The cell suspension was inverted every 30 min during the incubation. At the end of the incubation the cells were processed through the flow cytometer.

Flow Cytometry and Sorting

Cell samples stained with chromomycin A3 were analyzed using the Lawrence Livermore Laboratory FCM described by Van Dilla et al.$^{22}$ During flow cytometry the cells were forced to flow one at a time through a laser beam from an argon-ion laser (Spectra Physics 164-05) adjusted to emit 200 mw at 457 nm. The fluorescence from each cell was collected by a microscope objective and projected through a Corning CS 3-71 colored glass filter (to exclude scattered laser light) onto a photomultiplier to produce an electrical pulse that, when integrated, was proportional in amplitude to the total cellular fluorescence. The pulses from about 10$^3$ cells/sec were digitized and stored in a pulse-height analyzer to form a frequency distribution of cellular fluorescence (DNA-related fluorescence).

Flow sorting of chromomycin A3- and Hoechst-33342-stained cells was accomplished using either the Livermore FCM or a modified Becton & Dickinson FACS II described by Herzenberg et al.$^{23}$ Hoechst-33342-stained cells were jetted one at a time into air through a 100-μm-diameter orifice where they were illuminated with a laser beam from an argon-ion laser (Spectra Physics 164-05) adjusted to emit 100 mw at 351 and 364 nm. The resulting fluorescence was collected by a microscope objective and projected through a 450-nm high-pass filter (to exclude scattered laser light) onto a photomultiplier. The resulting electrical pulses (proportional to cellular fluorescence) were used to distinguish the cells to be sorted. A fluorescence distribution was generated by pulse-height analysis as described previously. After fluorescence analysis the cells continued down the liquid jet to the point where it broke into droplets; the droplets containing the cells to be sorted were electrically charged as they broke off from the liquid jet. The charged droplets were separated from the others during passage through a high-voltage electrical field and were collected on glass microscope slides for subsequent processing.

RESULTS

Separation of Megakaryocytes by CE

The results of cell counts and megakaryocyte counts from seven separate elutriations of mouse BM are shown in Table 1. It can be seen that the majority (68%) of cells sedimented at less than 10 mm/hr and thus passed through the rotor initially. This fraction contained approximately 3% of the input megakaryocytes. An additional small percentage (3.6%) of nucleated cells was removed in the wash along with about 0.7% of the input megakaryocytes. The fraction sedimenting at more than 10 mm/hr contained only 1.6% of the input nucleated cells but 27% of the input megakaryocytes.
Table 1. Concentration of Megakaryocytes by CE*

<table>
<thead>
<tr>
<th>Elutriator Fraction</th>
<th>Nucleated BM Cells</th>
<th>Megakaryocytes</th>
<th>Percentage Fraction</th>
<th>Concentration Factor of Input</th>
</tr>
</thead>
<tbody>
<tr>
<td>Input</td>
<td>2.8 × 10^6</td>
<td>100</td>
<td>1.4 × 10^3</td>
<td>5.0 × 10^{-4}</td>
</tr>
<tr>
<td></td>
<td>(0.7–5.6)</td>
<td></td>
<td>(0.2–2.4)</td>
<td>(2.8–6.5)</td>
</tr>
<tr>
<td>&lt; 10 mm/hr</td>
<td>1.9 × 10^6</td>
<td>69</td>
<td>4.6 × 10^2</td>
<td>2.6 × 10^{-5}</td>
</tr>
<tr>
<td></td>
<td>(0.4–3.0)</td>
<td>(50–120)</td>
<td>(1.0–9.2)</td>
<td>(0.6–4.0)</td>
</tr>
<tr>
<td>'Wash'</td>
<td>1.3 × 10^7</td>
<td>3.2</td>
<td>1.4 × 10^3</td>
<td>1.4 × 10^{-6}</td>
</tr>
<tr>
<td></td>
<td>(0.5–2.7)</td>
<td>(1.8–4.8)</td>
<td>(0.24–3.8)</td>
<td>(0.1–5.2)</td>
</tr>
<tr>
<td>&gt; 10 mm/hr</td>
<td>3.9 × 10^6</td>
<td>1.6</td>
<td>3.9 × 10^6</td>
<td>1.0 × 10^{-3}</td>
</tr>
<tr>
<td></td>
<td>(1.4–5.6)</td>
<td>(0.6–1.9)</td>
<td>(1.5–5.4)</td>
<td>(0.7–1.2)</td>
</tr>
</tbody>
</table>

*Values presented are the mean and range of six separate runs except for the ‘washes,’ which is the result of three runs.

A considerable concentration of megakaryocytes (approximately 24-fold) over the initial marrow sample was obtained by this procedure. One problem we encountered was aggregation of marrow cells at high input concentrations; this resulted in a significant fraction of megakaryocytes appearing in a pelleted fraction. This was overcome by decreasing the input concentration from about 5 × 10^6 nucleated cells/ml to 10^6 cells/ml.

Photomicrographs (Fig. 1) of the initial fraction and the fraction sedimenting at more than 10 mm/hr substantiate this enrichment and indicate good morphologic preservation following CE. Although they are not shown, similar results were obtained with rat marrow megakaryocytes under conditions identical to those described for mouse marrow.

Fluorescence Distributions

A fluorescence distribution of chromomycin-A3-stained mouse BM cells is shown in Fig. 2A. All DNA distributions shown in this report are plotted on a semilogarithmic scale so that the megakaryocytes can be more easily discerned; a cutoff of 10 cells per channel was selected. A number of peaks are evident, and the peak channel plus an estimate of the fractional area are presented in Table 2. The data from two other runs are also shown. The fluorescence peak indicative of diploid (2C) cells is the largest and accounts for about 70% of the cells. Next is the 4C peak containing about 20% of the cells. The peaks in the 6C and 8C positions account for about 2% and 1% of the cells, respectively. The 6C peak as well as the smaller 10C peak are probably artifacts of the flow cytometry technique (see Discussion). A relatively prominent peak containing about 3% of the cells is noted at the 16C position, with a smaller peak containing less than 1% of the cells appearing at 32C.

A distribution for CE-enriched cells stained with Hoechst 33342 is shown in Fig. 2B. The same peaks observed in the previous chromomycin A3 studies were also discerned here, although their distribution appeared to be broader. Analysis of this distribution (Table 2) indicated that it was similar to that obtained for chromomycin A3.

Finally, a fluorescence distribution was obtained for elutriated rat marrow stained with chromomycin A3. The distribution of rat marrow DNA-related...
Fig. 1. Photomicrograph of Sayk preparations of (A) starting mouse BM preparation and (B) the fraction sedimenting at more than 10 mm/hr after CE showing concentration of megakaryocytes. Stained with AChE (×125).

fluorescence (Fig. 2C) and its analysis (Table 2) indicated a close similarity to that obtained with mouse marrow.

**Microscopic Studies**

During FCM analysis, cells from various peaks were sorted onto “albuminized” slides that had been dipped in 0.5% BSA in PBS and air-dried. Hoechst 33342 was used as the DNA stain when subsequent staining for AChE activity was performed,
since viable cells were necessary prior to fixation in glutaraldehyde. Cells were stained with hematoxylin only when chromomycin A3 was used on ethanol-fixed preparations. Sorting results were similar to those described for Hoechst 33342. The total number of cells on each slide was counted at a magnification of 1000, and the results for one run of mouse marrow are presented in Fig. 3 and Table 3. The 2C peak (Fig. 3A) was composed of small cells of which 0.3% stained for AChE; the latter were very small megakaryocytes that were morphologically identifiable either with or without AChE staining. In general, nucleated cells in the 4C peak (Fig. 3B) were larger than those in the 2C peak, with a larger percentage of them being megakaryocytes (3.4%). The 8C, 16C, and 32C peaks (Figs. 3C, 3D, and 3E) contained increasingly pure populations of AChE-positive megakaryocytes, with those in the 32C peak comprising 84.4% of the total cells present on the slides. It was also clear that although megakaryocytes in the various fluorescence classes displayed a range of cell size and morphology, in general, those in the higher fluorescence classes were progressively larger in size and more mature, as judged by the degree of cytoplasmic granularity, as shown in Fig. 3 (C–F). In addition, progressively more “naked” megakaryocyte nuclei (Fig. 3F) were observed in the higher DNA-related fluorescence classes (6.4%, 18%, and 39% of megakaryocytes in 8C, 16C, and 32C classes, respectively).

Analysis of megakaryocytes in the different DNA-related fluorescence classes from the described run (Fig. 2B) was carried out by first obtaining the products “percentage cells in peak region” and “fraction megakaryocytes in each fluorescent class” and then using the sum of these products to determine the total frequency of occurrence of megakaryocytes. The “normalized” megakaryocytes in each class were then expressed as a percentage of this total. As shown in Table 4, over 75% of the megakaryocytes were nearly equally divided in the 4C and 16C peak regions.
Fig. 2. See caption on facing page.
DISCUSSION

This is the first published study in which megakaryocytes have been analyzed by FCM and sorted onto slides for subsequent microscopic evaluation. Two different types of stains were used: chromomycin A3, which produced good fluorescence distributions, and Hoechst 33342, which produced broader distributions, but in which the cells were viable and could be treated further (e.g., stained for AChE activity). The differences in the fluorescence distributions with the two stains may reflect somewhat their incomplete specificity for DNA (Hoechst 33342 having a different nonspecific mode than chromomycin A3), so that bare nuclei stain differently than cells with abundant cytoplasm. This would explain the broad 16C and 32C peaks. Furthermore, the stoichiometries of the two dyes are different, with Hoechst 33342 being adenine-thymine-specific and chromomycin A3 being guanine-cytosine-specific.

One difficulty encountered with FCM studies of megakaryocytes using unfractionated marrow is low frequency of the target cells (approximately 0.05%) coupled with the maximum rate of analysis of individual cells of $5 \times 10^3$ per second by present FCM technology. With an average 24-fold concentration of megakaryocytes by CE, however, we have demonstrated that enough target cells can be accumulated for subsequent analysis within a reasonable period of time. For example, collection of 100 32C megakaryocytes required 30–60 min of operation of the flow cytometer.
Fig. 3. See caption on page 742.
Fig. 3. See caption on page 742.
The first portion of the study dealt with the concentration of megakaryocytes from marrow so that subsequent FCM analysis could be accomplished. Although about 90% of the recovered megakaryocytes were in the fraction sedimenting at more than 10 mm/hr, only about 30% of the input megakaryocytes could be accounted for. Since the morphology of the CE-enriched megakaryocytes was well maintained and no obvious discrepancy in the ratio of immature to mature megakaryocytes was observed between those separated by CE and those in unfractionated marrow, we have assumed that these are representative of the population. It may be that the megakaryocytes that are lost are either “stickier” or “more fragile.” Further studies will deal with procedures to increase this recovery in the light of discrepancies in megakaryocyte recovery using other separation techniques.13-5 Our best CE procedure to date results in 73% of the input megakaryocytes being retained in the 4-ml rotor volume, with a 36-fold increase in their concentration such that they account for about 1% of the nucleated cells;
Table 3. Analysis of Cell-Sorted, CE-Enriched Mouse Marrow*

<table>
<thead>
<tr>
<th>Designated Fluorescence Class</th>
<th>Nucleated BM Cells per Slide</th>
<th>Megakaryocytes per Slide</th>
<th>Percentage Megakaryocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>2C</td>
<td>4739</td>
<td>14</td>
<td>0.3</td>
</tr>
<tr>
<td>4C</td>
<td>713</td>
<td>24</td>
<td>3.4</td>
</tr>
<tr>
<td>8C</td>
<td>203</td>
<td>44</td>
<td>21.6</td>
</tr>
<tr>
<td>16C</td>
<td>739</td>
<td>414</td>
<td>56.0</td>
</tr>
<tr>
<td>32C</td>
<td>238</td>
<td>201</td>
<td>84.4</td>
</tr>
</tbody>
</table>

*From Fig. 2B.

although obviously not constituting a “pure” fraction, they were sufficiently concentrated that FCM studies could be carried out.

The advantages of CE are the speed at which the concentrated sample is obtained (about 14 min), the minimal manipulation of the target cells, and the ability to wash the cells or change the suspending medium in the rotor without cell packing and resuspension.

It is expected that further modification of the procedure or addition of other steps such as density centrifugation will provide an even greater degree of concentration and an increased recovery of megakaryocytes.

Staining of the fraction sedimenting at more than 10 mm/hr with chromomycin A3 and analysis by FCM yielded a distribution with a number of peaks. Discernible in the distribution of both mouse and rat marrow were 2C, 4C, “6C,” 8C, “10C,” 16C, and 32C populations. As expected, the 2C and 4C peaks represented the vast majority (about 90%) of the cells in the starting cell population. Megakaryocytes were present in all the peaks, although they constituted a greater proportion of the cells in classes of increasing DNA-related fluorescent content. Although megakaryocytes were also present in the “6C” and “10C” peaks, we believe that these peaks are probably due to cell clumping. We plan to investigate this possibility more thoroughly by analyzing individual cell-containing droplets on slides using a moving stage and then determining the number and morphology of cells present in each droplet.

Megakaryocytes present in higher DNA-related fluorescence classes were both larger and morphologically more mature. The latter observation was supported by the higher incidence of “naked” megakaryocyte nuclei found in these same classes.
which Odell et al.\textsuperscript{8} have described as being indicative of the end stage of platelet production.

The distribution of mouse marrow megakaryocytes in the 8C, 16C, and 32C classes obtained by flow cytometry (Table 4) compared favorably with that for rat megakaryocytes obtained by scanning microspectrophotometry of Feulgen-stained marrow slide preparations,\textsuperscript{7} although we detected a substantially larger proportion of megakaryocytes in the 4C peak. This may reflect a species difference. However, difficulties inherent in the latter techniques in unequivocally identifying small numbers of megakaryocytes with relatively lower DNA contents may account for the lower proportions obtained by scanning microspectrophotometry. It may be difficult to identify such cells without a specific second marker such as AChE activity, although this also has its limitations, since Jackson\textsuperscript{24} has demonstrated that immature but morphologically identifiable rat megakaryocytes have substantially lower AChE activities than mature megakaryocytes.

The ability to combine CE and FCM techniques holds potentially great promise for elucidating the kinetics of megakaryocyte production and its regulation. Determination of the DNA-related fluorescence of the progenitor class, the existence and quantitation of S-phase cells and their morphologic definition, and the effects of agents that perturb the system (e.g., hormones and drugs) are a few of the many questions that can be studied.

REFERENCES

10. Weste SA, Pennington DG: Fluorometric measurement of deoxyribonucleic acid in bone marrow cells: The measurement of megakaryocyte deoxyribonucleic acid. J Histochem Cytochem 20:627, 1972
17. Nakeff A, Dicke KA, van Noord MJ: Megakaryocytes in agar cultures of mouse marrow, Ser Haematol 8:1, 1975
FLOW CYTOMETRY AND MEGAKARYOCYTOPOIESIS


Application of flow cytometry and cell sorting to megakaryocytogenesis

A Nakeff, F Valeriote, JW Gray and RJ Grabske