Immature Megakaryocytes in the Mouse: Morphology and Quantitation by Acetylcholinesterase Staining

By Michael W. Long and Neil Williams

Three types of immature megakaryocytes, detected by their morphological properties, have been characterized in bone marrow of normal C57BL/6 mice. Morphological classification of these cells was carried out by determining (1) presence and relative amount of acetylcholinesterase, (2) cell size, (3) nuclear/cytoplasm ratio, and (4) nuclear shape. The immature megakaryocytes were classified as: (A) cells distinguished by a round nucleus (10.6 ± 1.1 μm diameter; mean ± SEM), which had the highest nucleus/cytoplasm ratio and lowest content of acetylcholinesterase; (B) cells with an indented nucleus (13.0 ± 1.9 μm diameter), which had increased acetylcholinesterase content and reduced nucleus/cytoplasm ratio compared to the round-nucleus cell type; and (C) lobed-nucleus cells (14.5 ± 2.9 μm diameter), which showed further increase in acetylcholinesterase content and reduction in nucleus/cytoplasm ratio. Increased numbers of immature megakaryocytes were detected, indicating that a proportion of these cells are undetectable using conventional staining techniques. Based on the observed alterations in size, acetylcholinesterase content, and nuclear complexity, it was concluded that these cells constitute part of a progressive maturation sequence intermediate between the progenitor cell (CFU-Mk) and mature easily recognizable megakaryocytes.

Bone marrow megakaryocytes are recognizable by light microscopy by their large size and Romanovsky staining characteristics. Three stages are identifiable as: (1) megakaryoblasts (stage I) with an agranular, intensely basophilic cytoplasm, a lobed or clefted nucleus, and a high nucleus-to-cytoplasm ratio, (2) promegakaryocytes (stage II), changed only by scant-to-moderate amounts of azurophilic granules in the cytoplasmic and reduced nucleus/cytoplasm ratio, (3) the platelet-forming megakaryocyte (stage III), having an azurophilic granular cytoplasm, condensed multilobed nucleus, and the lowest nucleus/cytoplasm ratio.

In addition to these morphologically recognizable megakaryocytes, a population of smaller cells not recognizable by the above criteria may also belong to the megakaryocytic compartment. These small megakaryocytes are regarded as being immature among the megakaryocyte lineage based on their small size and the maturation of these cells into single megakaryocytes when stimulated with thrombopoietic stimulatory factor. The importance of the immature megakaryocytes in the overall regulatory process is suggested by their responsiveness to perturbations of in vivo megakaryopoiesis. Jackson and Nakeff found these small acetylcholinesterase-positive cells to be elevated within 6 hr following the administration of rabbit anti-mouse platelet sera. Similar changes were observed following the administration of thrombopoietic stimulatory factor to mice. In both cases, the elevation of these immature megakaryocytes occurred prior to changes in morphologically recognizable megakaryocytes. Immature megakaryocytes also appear to be the least mature of detectable megakaryocytes to respond to feedback regulation. These cells, but not the clonable progenitor cells, are suppressed following transfusion-induced thrombocytosis.

Since the initial studies of Jackson, little information has been obtained concerning the role of these cells in normal marrow. In that study, small acetylcholinesterase-containing bone marrow cells were described as a single population of cells. In the present study, quantitation of these immature megakaryocytes has been readdressed using a system that allows greatly enhanced detection. This cell population is reported, for the first time, to be heterogeneous, based on alterations in enzyme content, size, nucleus-to-cytoplasm ratio, and nuclear shape. The detection, quantitation, and morphological criteria for these
IMMATURE MEGAKARYOCYTES

subclasses of immature megakaryocytes in normal bone marrow are reported.

MATERIALS AND METHODS

Bone Marrow Cell Preparations

The C57BL/6 male mice (Cumberland View Farms, Tenn.), 4-6 mo old, were used in these studies. The content of both femurs was flushed into 10.0 ml of CATCH medium and monodispersed by gentle pipetting with a 5.0-ml plastic pipet. One-hundred microliters of bone marrow suspension containing 1.0-3.0 x 10^6 cells were loaded into the sample chamber of a cytocentrifuge (Sandoz, Sewicklen, Pa.) and spun for 3 min at 40 g onto acid-alcohol cleaned slides. At the end of this initial spin, a wash of 200 µl of fresh CATCH media was loaded into the sample chamber and the specimens were spun for an additional 10 min at 40 g. The slides were then removed and immediately air dried. The wash step results in good recovery of total cells and megakaryocytes on the slide. The cells were heat-fixed (50°C for 5 min) on a slide warmer and stained for acetylcholinesterase activity.

Acetylcholinesterase Staining

Acetylcholinesterase is a specific cytochemical marker for the megakaryocytic lineage in mice and certain other species. Acetylcholinesterase activity was detected using the method of Karnovsky and Roots as modified by Jackson. The cells were incubated in acetylthiocholine substrate for 6.0-6.5 hr. The substrate was prepared as follows: 10 mg of acetylthiocholine iodide (Sigma, St. Louis, Mo.) was dissolved in 15.0 ml of 0.1 M dibasic sodium phosphate (pH 6.0). To this the following were added, in order, with constant mixing: 1.0 ml of 0.5 M sodium citrate; 2.0 ml of 30 mM cupric sulfate; 2.0 ml of 5 mM potassium ferrocyanide. In order to detect cells with low acetylcholinesterase content, all reagents had to be freshly prepared and the reaction period was critical. Following reaction in acetylthiocholione substrate, the slides were fixed for 10 min in 95% EtOH, counterstained with Harris' hematoxylin (30 sec), washed in running tap water, blued in saturated lithium carbonate (20 sec), washed again, and air dried.

It was found that the staining time was critical for detection of the smaller acetylcholinesterase-positive cells. A reaction time of 6.0-6.5 hr resulted in the maximal detection of smaller immature megakaryocytes without changes in the numbers of large mature megakaryocytes. Other bone marrow cells, cultured hematopoietic cells, or cell lines were routinely negative for acetylcholinesterase activity (data not shown). Staining for 3.5-4.0 hr resulted in detection of 100% of the larger, mature megakaryocytes, whereas only 30-60% of the immature megakaryocytes were positive. Times of greater than 7 hr showed marked decrease in numbers of both cell types, most likely caused by elution of the cells from the slide. No change in WBC differential counts (Romanovsky stain) occurred between input and cytocentrifuge specimens, and good recovery (90%-100%) in the number of mature megakaryocytes was seen between input and collected samples (starting levels of megakaryocytes were counted by acetylcholinesterase-stained 10 µl smears of the suspension to be cytocentrifuged). The use of the cytocentrifuge preparations combined with phase contrast microscopy greatly improved detection of immature megakaryocytes in unfractoned bone marrow samples.

Examination of Acetylcholinesterase-Containing Bone Marrow Megakaryocytes

Murine acetylcholinesterase-containing megakaryocytes examined under phase contrast microscopy (oil, Zeiss Planapo Ph 3, 40x objective) were classified according to the following criteria: (1) acetylcholinesterase content, (2) cell size, (3) nucleus/cytoplasm ratio, (4) nuclear shape. Acetylcholinesterase activity was recognizable by the presence of granular deposits of copper ferrocyanide (Hatchett's Brown), the ultimate reaction products of the acetylcholinesterase stain. Under phase contrast, these granules appeared black in those cells with low acetylcholinesterase content and orange-black in the intensely stained larger megakaryocytes.

Nuclear configurations were recorded as round, indented, or lobed shapes. The nucleus-to-cytoplasm ratio was estimated based on relative nuclear size. Differential types of acetylcholinesterase-containing megakaryocytes were scored and 200-250 cells/slide recorded for each of 20 animals.

Following morphological classification, 1500 immature megakaryocytes meeting the above criteria were sized using an ocular sizing eyepiece (Zeiss), which was calibrated using a metric stage micrometer (American Optical). Cells were chosen for sizing that were free of cell-to-cell contact. Irregularly shaped cells were recorded as the average of the long and short axis.

In addition to acetylcholinesterase staining, parallel preparations of bone marrow cells were stained for routine morphological analysis using May-Gruenewald-Giemsa stain. The marrow megakaryocytes were identified and 100 megakaryoblasts (stage 1) sized.

Assessment of Acetylcholinesterase Content

Enriched populations of immature megakaryocytes, obtained by velocity sedimentation, were analyzed for relative acetylcholinesterase content using an Artek Image Analyzer (made available by the courtesy of Dr. Paul Bunn, NCI-VA Medical Branch, Veterans Administration Medical Center, Washington, D.C.). Acetylcholinesterase-positive cells were identified using the above criteria and optical density measured without uncounterstaining the cells. Acetylcholinesterase content is expressed in arbitrary optical density units integrated from the entire cell area. The Artek analyzer was used in a mode that automatically adjusts for changes in background density. Using this technique, cells negative for acetylcholinesterase gave readings of <1.0 U.

RESULTS

Morphological Classification of Acetylcholinesterase-Containing Bone Marrow Megakaryocytes

Immature megakaryocytes were classified according to acetylcholinesterase content, size, nuclear shape, and nucleus-to-cytoplasm ratio. The cells could be directly identified by phase contrast microscopy. They were recognizably smaller than mature megakaryocytes and contained reduced amounts of acetylcholinesterase.

Within this small megakaryocyte classification, three morphological cell types could be identified. They were readily distinguished from mature megakaryocytes (≥18 µ diameter) by four criteria: (1) a markedly less intense acetylcholinesterase reaction; (2) a relatively small size with respect to mature megakaryocytes (i.e., 8-18 µ in diameter); (3) a high nucleus-to-cytoplasm ratio; and (4) distinct nuclear shapes: round, indented, or lobed nuclei.

Of the three morphological types identified, the
Fig. 1. Murine marrow megakaryocytes (phase contrast). Marrow cells stained for acetylcholinesterase. Acetylcholinesterase activity is shown as black granular deposits of copper ferrocyanide seen in the cytoplasm. (A) Immature megakaryocyte: round nucleus type; (B) immature megakaryocytes: indented nucleus type; (C) immature megakaryocyte: lobed nucleus type; (D) mature megakaryocyte: multilobulated nucleus (35 x).
least frequently observed cell is seen in Fig. 1A. This cell is characterized by a round nucleus showing little chromatin, a high (>1.0) nucleus-to-cytoplasm ratio, and scanty amounts of acetylcholinesterase in the cytoplasm. These cells comprised 1.5% ± 0.8% of the total detectable megakaryocyte compartment and 1.8% ± 0.8% of the small immature megakaryocytes (n = 41; mean ± SEM) (Table 1).

The next frequent type was characterized by an indented nucleus and by a (subjectively) greater acetylcholinesterase content judged on an apparent increase in the number and size of copper ferrocyanide deposits (Fig. 1B). This cell type comprised 3.4% ± 0.6% of the total acetylcholinesterase-positive megakaryocytes and 3.7% ± 2.0% of the smaller cells. The indentation of the nucleus was reminiscent of that of the metamyelocyte/band configuration seen in the granulocyte series (n = 232; mean ± SEM).

The most prevalent type is shown in Fig. 1C. These cells have a clefted or lobulated nuclear configuration, showing what appears to be nuclear condensation. Compared to the previous two cell types, the lobed nucleus cell had the greatest apparent acetylcholinesterase content. This morphology makes up 68.5% ± 1.04% of the total megakaryocytes and represents 94.4% ± 10.0% of the immature cell compartment (n = 727; mean ± SEM).

Figure 1D shows a large, mature megakaryocyte. It is distinguishable from the earlier cell types by (1) the large size (28.5 ± 10 μm); (2) very intense acetylcholinesterase reaction, and/or (3) a low nucleus/cytoplasm ratio (<=1.0). This grouping of cells was thought to contain all three stages (I–III) of megakaryocytes recognizable by Romanovsky staining characteristics. However, distinction of these megakaryocyte types was not possible, due to the intensity of the acetylcholinesterase reaction (n = 498; mean ± SEM).

The lower amounts of the orange-brown acetylcholinesterase reaction product ( Hatchett’s Brown) in the cytoplasm of some immature megakaryocytes made their ready recognition difficult and they could not be objectively discriminated above the other marrow cells by bright field microscopy. Many of these latter cells also had a slight brown appearance. Distinction of true copper ferrocyanide deposition in the immature megakaryocytes could only be confidently determined by phase contrast microscopy. It should be noted that the granules of reaction product were not present when the cells were reacted in a substrate solution deficient in acetylthiocholine.

While false-positive ACh-E reactions have yet to be reported, bone marrow reticulum cells are known to contain hemosiderin, deposits of which appear golden. However, the morphology of reticulum cells allows them to be easily distinguished from small acetylcholinesterase-positive cells. Figure 2A shows a murine reticulum cell, a large cell with very low nucleus/cytoplasm ratio and a condensed eccentric nucleus (small arrow) (phase contrast; unstained bone marrow). Figure 2B shows a reticulum cell (small arrow) incubated in acetylcholinesterase substrate as it appears on a cytocentrifuge preparation. Figure 2C compares another reticulum cell (small arrow) with a lobed type of immature megakaryocyte (large arrow).

### Cell Size

The size distribution of the immature megakaryocyte cell types (Fig. 3) were markedly narrower than that of the mature, easily recognizable megakaryocytes (Fig. 4). Within the immature megakaryocyte classification, a progressively increasing modal size was obtained from the round nucleus type (11 μm), to indented nucleus (13 μm) and lobed nucleus (17 μm). The lobed nucleus form of immature megakaryocyte had a bimodal distribution (13 μm and 17 μm) that overlapped with the smaller of the mature megakaryocytes (Fig. 4).

The cells characterized by a round nucleus measured 10.6 ± 1.1 μm in diameter (mean ± SD), indented nucleus 13.0 ± 1.9 μm in diameter, and lobulated nucleus 14.5 ± 2.9 μm in diameter. While the mean value in each class increased, no significant difference in cell diameter was detectable among these cells when tested at the p ≤ 0.05 level of significance (Student’s t test). However, the largest of the small immature megakaryocytes (lobed nucleus, 14.5 ± 2.9 μm) was significantly smaller than the large mature megakaryocytes (28.5 ± 10.4 μm; p ≤ 0.05).

These cells were sized on cytocentrifuged preparations that tend to “spread out” the cells. Conversely, staining and fixing have been shown to decrease cell size. As the measured size of mature megakaryocytes of these stained, cytocentrifuged preparations were identical to that reported elsewhere, no size corrections were performed.

Sizing of megakaryoblasts (stage I) indicated that these cells overlapped the size distribution of the lobed table:

<table>
<thead>
<tr>
<th>Table 1. Morphological Comparison Between the Lobed Nucleus Acetylcholinesterase-Containing Immature Megakaryocytes and Megakaryoblasts (Stage I)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lobed Nucleus (Acetylcholinesterase)</td>
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<tr>
<td>--------------------------------</td>
</tr>
<tr>
<td>Size (microns ± SEM)</td>
</tr>
<tr>
<td>Nucleus-to-cytoplasm ratio</td>
</tr>
<tr>
<td>Nuclear shape</td>
</tr>
</tbody>
</table>

Fig. 2. Murine bone marrow reticulum cells (phase contrast). (A) Reticulum cell (small arrow); unstained specimen; (B) reticulum cell following incubation in acetylcholinesterase substitute (small arrow); (C) reticulum cell (small arrow) and lobed form of murine immature megakaryocyte (large arrow). B and C were cytocentrifuged and stained for acetylcholinesterase.
The size of the megakaryoblasts was seen to be \(15.5 \pm 2.6 \mu\) microns, whereas the lobed acetylcholinesterase-positive cell measured \(14.5 \pm 2.9 \mu\) in diameter.

**Acetylcholinesterase Content**

Measurements of acetylcholinesterase were performed by image analysis. Measurement of integrated optical density in single acetylcholinesterase-positive cells showed a progressive increase in copper ferrocyanide deposits from round nucleus through lobed nucleus cell types. This confirmed the subjective, visual evaluation reached during the studies using phase contrast microscopy. The integrated optical densities as measurement in arbitrary units were: round, \(4.8 \pm 2.2\) U (mean \(\pm\) SD), indented, \(6.4 \pm 2.9\) U, and lobulated, \(11.0 \pm 3.9\) U. As with the cell size, a progressive increase in the cellular content of reaction product was seen without statistical difference among the three types. The large mature megakaryocytes had an optical density of \(4536 \pm 208\) arbitrary units.

**DISCUSSION**

In previous reports, a proportion of the megakaryocyte compartment was presented as a population of small acetylcholinesterase-positive cells.4,8,17 In this report, these immature megakaryocytes were shown to be a heterogeneous population of three morphological cell types. The identification of these cells was facilitated by optimization of the detection of acetylcholinesterase-containing cells, as well as differential analysis of various cell parameters: (1) acetylcholinesterase content; (2) cell size; (3) nucleus/cytoplasm ratio; and (4) nuclear shape.

Certain similarities exist between the murine immature megakaryocytes and the analogous population of cells observed in human marrow. The human cells bear megakaryocyte-platelet protein markers and have a similar incidence.6,10,12,14 Levine has reported that the size distribution of smaller, younger human megakaryocytes is 10–24 \(\mu\) microns, which is comparable with the range reported here for murine cells.4 Further the human immature megakaryocytes were characterized by an irregular nuclear outline (and lobes), high nucleus-to-cytoplasmic ratio, and a progressive increase in granular content and cytoplasm.4

Early studies placed the rodent small acetylcholinesterase-containing cells as less differentiated, but related to megakaryocytes based on the presence of cytochemical marker, their small size (with respect to mature megakaryocytes), and similar properties.2,7,11,22 Such immature megakaryocytes can also be distinguished from the megakaryocyte progenitor cell...
(CFU-Mk), since cells containing acetylcholinesterase are well separated from the progenitor cells.\textsuperscript{5,9} Moreover, separated populations of these immature cells mature into large single megakaryocytes but lack the ability to proliferate (even into two-cell colonies).\textsuperscript{3} The observation of a progressive increase in acetylcholinesterase content, cell size (8–18 \(\mu\text{m}\)), and nuclear complexity among these three cell types supports and extends the hypothesis that these cells are intermediate between megakaryocyte progenitor cells (CFU-Mk) and larger, mature megakaryocytes.\textsuperscript{2,3,8,16,17}

Comparisons of the lobed nucleus acetylcholinesterase-containing cells with megakaryoblasts (stage I megakaryocytes) indicate that the size range of these two populations overlap. Also, nuclear shape and nucleus-to-cytoplasms ratios are similar (Table 1). In a subsequent study, we show that megakaryoblasts and promegakaryocytes classified by Romanovsky staining criteria are found to make up a majority of the cells described here as lobed cells.\textsuperscript{23} Therefore, these may be the same population of cells, differing only in detection. This increase in detection, using acetylcholinesterase and phase contrast microscopy, results in detection of approximately 22,000 detectable megakaryocytes per femur (Table 2), whereas reports of megakaryocyte frequency determined by Romanovsky staining indicated approximately 15,000/femur.\textsuperscript{24} Thus, determination of megakaryocyte numbers by acetylcholinesterase content and nuclear morphology redistributes the megakaryocyte compartment described by Romanovsky criteria into high numbers of immature and low numbers of mature megakaryocytes. This alternative view suggests new, but speculative, relationships concerning the cellular megakaryocyte stage can be regulated. Negative feedback regulation is implied from observations that immature megakaryocytes,\textsuperscript{2} but not the progenitor cells,\textsuperscript{17,25} are sensitive to platelet-derived feedback mechanisms. This steady-state feedback regulation can be modulated by administration of thrombopoietic stimulatory factor or anti-mouse platelet antisera, resulting in elevation of the immature megakaryocytes.\textsuperscript{2,8,15,16,26} However, other studies\textsuperscript{6,26} have not supported either the role of thrombopoietic stimulatory factor or immunothrombocytopenia production of thrombopoietin(s) in the regulation of thrombopoiesis so the nature of the regulator(s) remains unclear.

The accumulation of the lobed type of immature megakaryocytes suggests that the movement from immature to mature megakaryocyte may be another level of negative regulation. A large increase in size and cytoplasmic content occurs at the transition from immature to mature megakaryocytes, indicating that this is a point of positive control. Such changes in megakaryocyte volume have been observed to occur during perturbations of thrombopoiesis and precede alterations in megakaryocyte number.\textsuperscript{22,27,33}

Thus, the change in cell numbers, size, and acetylcholinesterase content between the progenitor cells and round nucleus immature megakaryocytes and the similar changes between the immature and mature megakaryocytes suggest that movement into and out of the immature megakaryocyte stage may be by independently regulated events.

**ACKNOWLEDGMENT**

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**Table 2. Characteristics of Murine Femoral Megakaryocyte Compartment as Determined by Acetylcholinesterase Staining and Phase Contrast Microscopy**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>CFU-Mk</th>
<th>Immature Megakaryocytes</th>
<th>Mature Megakaryocytes</th>
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<tbody>
<tr>
<td>Nuclear shape</td>
<td>NT</td>
<td>Round</td>
<td>Lobed</td>
</tr>
<tr>
<td>Nucleus/cytoplasm</td>
<td>NT</td>
<td>High</td>
<td>Low</td>
</tr>
<tr>
<td>ACh-E content</td>
<td>—</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Arbitrary OD</td>
<td>NT</td>
<td>4.8 ± 0.4</td>
<td>11.0 ± 0.9</td>
</tr>
<tr>
<td>Femur content</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Standard error</td>
<td>4,937 ± 201</td>
<td>310 ± 33.5</td>
<td>15,800 ± 1207</td>
</tr>
<tr>
<td>ACh-E-containing cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Percent of all megakaryocytes</td>
<td>1.5 ± 2</td>
<td>3.4 ± 0.1</td>
<td>68.5 ± 2</td>
</tr>
<tr>
<td>Percent of immature cells</td>
<td>1.8 ± 0.2</td>
<td>3.7 ± 0.4</td>
<td>94.4 ± 7</td>
</tr>
<tr>
<td>Size (microns)</td>
<td>7.0*</td>
<td>10.6 ± 0.2</td>
<td>13.0 ± 0.4</td>
</tr>
</tbody>
</table>

Values are mean ± SEM; NT, not tested; ACh-E, acetylcholinesterase.

REFERENCES

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