Method for Enrichment of Proliferating Myeloid Cells From Normal and Leukemic Human Bone Marrow


A method is described for the enrichment of immature myeloid cells from normal human bone marrow. The procedure consists of three consecutive steps: density gradient centrifugation, hypotonic shock, and velocity sedimentation. The results of 10 isolations are reported in this paper. The unprocessed bone marrow contained an average of 8.9% immature myeloid cells. Cell differential counts of interface fractions, obtained after density gradient (Ficoll–Isopaque 1.068 g/ml) centrifugation, revealed an average of 27.2% immature myeloid cells. Subsequently, erythroid precursors were removed by hypotonic shock. The average concentration of immature myeloid cells increased to 36.4%. After the final step, velocity sedimentation at 1 g, fractions were collected with an average of 62.6% immature myeloid cells, of which 25.4% were in S-phase. Bone marrow cells from five patients with full-blown (>85% blast cells) acute myeloid leukemia were subjected to the same procedure, excluding hypotonic shock. The mean percentage of S-phase cells in the unprocessed bone marrow was 9.7%. Interface fractions (Ficoll–Isopaque 1.074 g/ml) consisted of more than 90% leukemic myeloblasts, of which 9.8% (mean value) were in S-phase. After velocity sedimentation a separation was obtained in fractions with mean percentages of DNA-synthesizing cells ranging from 3.8% to 26.6%.

Several techniques are known for the isolation and separation of cells. Isolation of immature myeloid cells (myeloblasts, promyelocytes, myelocytes) from normal bone marrow has been performed either with density gradient centrifugation or with velocity sedimentation. Neither of these methods offers satisfactory results due to an overlap in cell sizes and cell densities of normal bone marrow cells.

In this paper the results of a combined application of density gradient centrifugation, hypotonic shock (in order to remove the majority of erythroid precursor cells), and velocity sedimentation are reported.

Human leukemic bone marrow, in contrast to normal bone marrow, is composed of a morphologically rather homogeneous cell population. The percentage of DNA-synthesizing leukemic cells, however, varies considerably among patients with acute myeloid leukemia. Velocity sedimentation has been used to separate cells in specific phases of the cell cycle. The method has also been applied for cytokinetic studies in spontaneous AKR murine leukemia. In this study velocity sedimentation has been used to separate human leukemic S-phase cells from cells not involved in DNA synthesis.

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MATERIALS AND METHODS

Patients

Normal bone marrow was obtained from 10 patients undergoing cardiac surgery. Leukemic marrow was obtained from 5 patients with full-blown acute myeloid leukemia. Normal as well as leukemic samples (1 ml) were mixed with 4 ml anticoagulant (buffered citrate-dextrose; pH 7.0, formula A, USP xv), diluted with a Krebs Ringer-Tris (KRT) solution containing 5% fetal calf serum (FCS), and filtered through a nylon filter (pore size 100 μm). The whole isolation procedure was carried out at 4°C in order to assure optimal cell viability as recommended by Cutts.

Density Gradient Centrifugation

For further processing, 10–15 × 10^7 normal bone marrow cells were used. Twenty-ml samples of KRT-FCS containing 2 × 10^7 normal bone marrow cells were layered on 10 ml of a Ficoll (molecular weight 400,000) Isopaque (in KRT-FCS) cushion with a density of 1.068 g/ml. Leukemic myeloblasts (2–4 × 10^6 bone marrow cells) were applied to a Ficoll Isopaque cushion with a density of 1.074 g/ml. Ficoll-Isopaque solutions were made as described by Loos and Roos. Isotonicity was verified by freezing point depression and densities were measured at 25°C with a pycnometer. The tubes were centrifuged at 1400 g (calculated at the interface) for 30 min at 4°C in a swinging bucket rotor (HL8A) of a Sorvall centrifuge (RC-3). Interface fractions were collected by puncturing the tube just below the visible band.

The cellular composition of the interface fraction was determined in six experiments. In three of these experiments, cells present in the cushion and pellet were collected separately. In each of these fractions (interface, cushion, pellet) counts of nucleated cells (Coulter Counter model ZF) and cell differential counts (cytocentrifuge smears) were made. Subsequently, the number of immature myeloid cells in each fraction was determined, and from these data the recovery of the immature myeloid cells at the interface could be calculated. The calculation of cell recovery was done in this way in order to exclude misinterpretations introduced by overall cell loss (mean cell loss 22.0%, range 6–33%). The number of leukemic cells was determined only in the interface fraction. The recovery of leukemic myeloblasts at the interface was expressed as a percentage of the applied number of leukemic cells.

Hypotonic Shock

Interface cells, obtained from normal bone marrow samples, were exposed to water for 30 sec. Isotonicity was then restored with hypertonic KRT. Hypotonic treatment was not done on leukemic interface cells because of the negligible number of normoblasts in these samples. Incorporation studies with interface cells from normal bone marrow were performed before and after hypotonic shock in order to determine the influence of water treatment on the metabolic activities of immature myeloid cells.

Incorporation Studies

Incorporation studies were performed in 1-ml samples with cell numbers varying from 10^3 to 5 × 10^5 cells. The cells were suspended in KRT with 20% AB plasma (v/v) and incubated for 60 min at 37°C in a shaking waterbath. Control cells were kept at 0°C. Per milliliter of sample, 1 μCi ³H-thymidine (specific activity, 5 Ci/m mole), 5 μCi ³H-uridine (specific activity, 28 Ci/m mole), or 5 μCi ³H-leucine (specific activity, 55 Ci/m mole) was added. After incubation the cells were placed on ice and 2 ml of cold KRT containing 0.25 mM 2-deoxy thymidine, 0.25 mM uridine, and 0.25 mM leucine were added. The cells were washed twice with this medium and subsequently three times with ice-cold 1 M HClO₄ and once with 96% ethanol. Hyamine (0.2 ml) was added and the tubes were kept at 37°C for 60 min. The samples were transferred to liquid scintillation vials with 4 ml of water, 6 ml of Instagel were added, and radioactivity was counted (LKB model 81000). Nucleic acid and protein synthesis were expressed in fmole ³H-thymidine, ³H-uridine, and ³H-leucine incorporated per 5 × 10^5 cells. A linear relationship was observed between cell number in the range mentioned above and the incorporation of radioactive precursors.
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Velocity Sedimentation

For velocity sedimentation\textsuperscript{15} at 1 g, \(10^7\) cells were used. The method has been described in detail by Burghouts et al.\textsuperscript{13} In brief, 150 ml of 15\% (w/v) Ficoll (molecular weight 70,000) in KRT supplemented with 2\% bovine serum albumin (BSA) was layered on the bottom of a square glass tube (length 50 cm, bottom surface 5 x 5 cm). Subsequently, 9 layers of 25 ml each were stacked in the gradient with Ficoll concentrations of 2.3\% - 10\% (w/v) in KRT-BSA. Two ml of cell suspension (in 1\% Ficoll KRT-BSA) containing \(10^7\) cells (either normal or leukemic) were placed on top of the gradient, and finally an overlay of 25 ml KRT-BSA was applied. The tube was tilted gradually to a horizontal position. This maneuver decreased the distance between the different gradient layers to one-tenth the distance in the vertical position,\textsuperscript{16} and provided a gain in time needed for sedimentation. After sedimentation for 45 min at 1 g the tube was rotated back and fractions of 7 ml were collected from the bottom. Cell counts were made in these fractions. Appropriate fractions (five in total) were pooled for DNA measurement by means of pulse cytophotometry and cytocentrifuge smears.

In each of the five fractions (Fig. 2) the number of immature myeloid cells was determined and the recovery of these cells in specific fractions (Fig. 2, fractions IV and V) could be calculated. This procedure was followed in six experiments. In four other experiments, in which only the fraction containing the highest percentage of immature myeloid cells was isolated, the first 200 ml of the gradient (including the 15\% Ficoll layer) were discarded and the following 50 ml were collected.

Pulse Cytophotometry

Nuclear DNA contents of \(5-10 \times 10^4\) separate cells in the different samples were measured by pulse cytophotometry. Cells were stained with 10 ml of ethidium bromide (25 \(\mu\)g/ml) dissolved in a hypotonic (0.1\%) citrate solution.\textsuperscript{17} DNA histograms were obtained with an ICP-II Impulse Cytophotometer (Phywe, Gottingen, West Germany). According to the nuclear DNA content, the percentages of cells in the different phases of the cell cycle (G\(_1\), postmitotic phase; S, DNA synthesis phase; G\(_2\), premitotic phase; M, mitotic phase) were estimated from DNA histograms.\textsuperscript{18,19}

Cytocentrifuge Smears

Cytocentrifuge smears were made from the unprocessed bone marrow, from the different fractions obtained after density gradient centrifugation (interface, cushion, pellet) and hypotonic shock, and from pooled samples collected after velocity sedimentation. Samples of \(5 \times 10^4\) cells in 0.1 ml of KRT 20\% (v/v) human plasma protein solution were applied to a Cytospin centrifuge (SCA 0030, Shandon Southern, Camberley, England) and rotated for 10 min at 14 g. For each determination 200-300 cells were counted independently by an experienced hematomorphologist (J.S.)

Chemicals

Chemicals were obtained as follows: FCS from Flow Laboratories, England; Ficoll from Pharmacia Fine Chemicals, Uppsala, Sweden; Isopaque from Nyegaard, Oslo, Norway; BAS from Sigma Chemicals, St. Louis, Mo.; human plasma protein from Red Cross Blood Bank, Amsterdam, The Netherlands; \(^3\)H-thymidine, \(^3\)H-uridine, and \(^3\)H-leucine from Radiochemical Centre, Amersham, England; Hyamine and Instagel from Packard Instruments, Downers Grove, Ill.; 2-deoxy-thymidine, uridine, and leucine from Merck, Darmstadt, West Germany; and ethidium-bromide from Serva Feinbiochemica, Heidelberg, West Germany.

RESULTS

Normal Bone Marrow

The isolation procedure was carried out with 10 normal bone marrow samples. The cellular composition of 4 unprocessed marrow samples was determined and is presented in Table 1. The percentage of immature myeloid cells in these samples (mean 8.9\%, myeloblasts 1.0\%, promyelocytes 1.2\%, myelo-
## Table 1. Cell Differential Counts (%) of Normal Bone Marrow Samples and Recoveries of Immature Myeloid Cells Obtained During a Three-Step Isolation Procedure

<table>
<thead>
<tr>
<th></th>
<th>Bone Marrow</th>
<th>Density Gradient (Interface)</th>
<th>Interface, Hypotonic Shock</th>
<th>Velocity Sedimentation*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myeloblasts</td>
<td>1.0 (0-2)</td>
<td>4.5 (2-6)</td>
<td>4.2 (2-6)</td>
<td>6.0 (1-15)</td>
</tr>
<tr>
<td>Promyelocytes</td>
<td>1.2 (1-2)</td>
<td>6.0 (1-10)</td>
<td>8.7 (4-19)</td>
<td>9.2 (3-15)</td>
</tr>
<tr>
<td>Myelocytes</td>
<td>6.7 (5-8)</td>
<td>16.7 (12-24)</td>
<td>23.5 (19-28)</td>
<td>35.5 (22-54)</td>
</tr>
<tr>
<td>Metamyelocytes</td>
<td>9.0 (7-10)</td>
<td>12.3 (7-16)</td>
<td>12.0 (7-18)</td>
<td>17.5 (8-30)</td>
</tr>
<tr>
<td>Bands and polymorphs</td>
<td>41.7 (33-48)</td>
<td>8.5 (1-30)</td>
<td>11.3 (4-28)</td>
<td>15.5 (8-30)</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>15.0 (12-19)</td>
<td>26.5 (9-37)</td>
<td>32.5 (13-48)</td>
<td>8.5 (1-17)</td>
</tr>
<tr>
<td>Plasma cells</td>
<td>0.5 (0-1)</td>
<td>1.3 (0-5)</td>
<td>0.7 (0-2)</td>
<td>1.5 (0-4)</td>
</tr>
<tr>
<td>Monocytes</td>
<td>1.5 (0-3)</td>
<td>3.0 (1-5)</td>
<td>2.0 (0-4)</td>
<td>1.8 (0-3)</td>
</tr>
<tr>
<td>Normoblasts</td>
<td>23.2 (19-29)</td>
<td>21.2 (11-31)</td>
<td>5.1 (3-6)</td>
<td>6.5 (1-12)</td>
</tr>
<tr>
<td>Recovery of immature myeloid cells (%)</td>
<td>43.3 (28-52)</td>
<td>96.2 (87-100)</td>
<td>39.2 (34-48)</td>
<td>33.5 (24-52)</td>
</tr>
</tbody>
</table>

Results are the averages of six experiments, with the exception of the cell differential counts of the unprocessed bone marrow, which was determined in four samples, and the recovery of immature myeloid cells after density gradient centrifugation, which was determined in three experiments. The range is shown in parenthesis.

*See Fig. 2.

cytes 6.7%, corresponded with data obtained from 9 other experiments (mean 9.8%, range 1%-15%).

### Density Gradient Centrifugation

The cellular composition of six interface fractions obtained after density gradient (Ficoll–Isopaque 1.068 g/ml) centrifugation is presented in Table 1. The interface consisted of 27.2% immature myeloid cells (myeloblasts 4.5%, promyelocytes 6.0%, myelocytes 16.7%) and 20.8% mature myeloid cells. The ratio of immature to mature myeloid cells was decreased by increasing the density of the Ficoll–Isopaque solutions. Decreasing the density resulted in lower recoveries of immature myeloid cells at the interface. The Ficoll–Isopaque cushion with a density of 1.068 g/ml gave the best results with respect to recovery of immature myeloid cells at the interface and least contamination with mature myeloid cells.

In three experiments the recovery of immature myeloid cells at the interface after density gradient (1.068 g/ml) centrifugation was determined. The mean recovery in these experiments was 43.3% (Table 1). This value corresponded well with recoveries observed in a series of 12 similar experiments described elsewhere. In the latter experiments 37.0% (range 25%-54%) of the immature myeloid cells were found in the interface fraction after density gradient (1.068 g/ml) centrifugation.

### Hypotonic Shock

Subsequently, interface cells were subjected to water exposure for 30 sec. As can be seen from Table 1, more than 75% of the normoblasts disappeared after water treatment, whereas the recovery of immature myeloid cells was 96.2%. On cell differential counts the percentage of immature myeloid cells in-
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Fig. 1. Influence of water treatment (30 min) on interface cells obtained after density (1.068 g/ml) gradient centrifugation of a normal bone marrow sample as determined with the incorporation of radioactive thymidine, uridine, and leucine. I, interface cells not treated with water; II, interface cells treated once with water; III, interface cells treated twice with water. Incorporation was calculated as f/mole/5 x 10^6 cells.

creased to 36.4% (myeloblasts 4.2%, promyelocytes 8.7%, myelocytes 23.5%). Of the erythrocytic series all cells were lysed by hypotonic shock with the exception of proerythroblasts.

In order to determine if water treatment influences the metabolic activities of the immature cells, the extent of incorporation of ^3H-thymidine, ^3H-uridine, and ^3H-leucine was investigated before and after water treatment. In each experiment incubations were performed with the same number of cells. Incubated samples obtained after lysis contained more immature myeloid cells and fewer erythroid precursor cells than untreated samples. This increase in immature myeloid cells apparently was responsible for the observation that the incorporation with labeled precursors did not change after hypotonic shock (Fig. 1). A second hypotonic shock did not alter the cellular composition of the sample, diminish the number of immature myeloid cells, nor affect the incorporation of radioactivity (Fig. 1). These experiments allowed the conclusion that the immature myeloid cells are apparently not damaged by water treatment.

Velocity Sedimentation

After water treatment, interface cells were applied to the gradient for velocity sedimentation at 1 g. The results of such a sedimentation are presented in Fig. 2. In six experiments fractions were pooled as presented in Fig. 2. The majority

<table>
<thead>
<tr>
<th>Fraction</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>V</th>
</tr>
</thead>
<tbody>
<tr>
<td>% immature myeloid cells</td>
<td>10</td>
<td>21</td>
<td>24</td>
<td>38</td>
<td>72</td>
</tr>
<tr>
<td>% G-phase cells</td>
<td>2.7</td>
<td>3.2</td>
<td>5.8</td>
<td>17.8</td>
<td>30.8</td>
</tr>
<tr>
<td>% G2 + M-phase cells</td>
<td>0.6</td>
<td>0.4</td>
<td>0.8</td>
<td>3.2</td>
<td>12.7</td>
</tr>
</tbody>
</table>

Fig. 2. Sedimentation (at 1 g) profile of normal bone marrow cells obtained after density gradient centrifugation and water treatment. Appropriate fractions (I-V) were pooled and the percentages of S-phase and G2 + M-phase cells were determined by pulse cytophotometry. Percentage of immature myeloid cells in these pooled fractions was determined on cytocentrifuge smears. Fraction V consisted of 72% immature myeloid cells, of which a high percentage was in S-phase (30.8%).
Table 2. Samples with High Percentages of Immature Myeloid Cells Obtained From Normal Bone Marrow

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>Myeloblasts (%)</th>
<th>Promyelocytes (%)</th>
<th>Myelocytes (%)</th>
<th>Phases (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>S</td>
</tr>
<tr>
<td>1</td>
<td>7</td>
<td>15</td>
<td>31</td>
<td>20.9</td>
</tr>
<tr>
<td>2</td>
<td>11</td>
<td>18</td>
<td>27</td>
<td>21.6</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>21</td>
<td>27</td>
<td>25.0</td>
</tr>
<tr>
<td>4</td>
<td>11</td>
<td>23</td>
<td>32</td>
<td>19.0</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>22</td>
<td>29</td>
<td>25.2</td>
</tr>
<tr>
<td>6</td>
<td>7</td>
<td>25</td>
<td>40</td>
<td>30.8</td>
</tr>
<tr>
<td>7</td>
<td>27</td>
<td>22</td>
<td>12</td>
<td>25.6</td>
</tr>
<tr>
<td>8</td>
<td>22</td>
<td>27</td>
<td>13</td>
<td>24.3</td>
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<tr>
<td>9</td>
<td>16</td>
<td>23</td>
<td>26</td>
<td>36.1</td>
</tr>
<tr>
<td>10</td>
<td>25</td>
<td>26</td>
<td>17</td>
<td>25.3</td>
</tr>
<tr>
<td>Mean</td>
<td>14.6*</td>
<td>22.2*</td>
<td>25.4*</td>
<td>25.4</td>
</tr>
</tbody>
</table>

Results of 10 isolations performed on normal bone marrow are summarized. Samples were obtained after density gradient centrifugation, hypotonic shock, and velocity sedimentation. In experiments 1–6 samples were identical to fraction V (Fig. 2). In experiments 7–10, samples were collected a bit differently, as described in Materials and Methods. Percentage of S-phase and G2 + M-phase cells was determined with pulse cytophotometry. The percentage of immature myeloid cells was determined on cytocentrifuge smears.

*Mean of myeloblasts + promyelocytes + myelocytes: 62.2%.

(72.7%) of immature myeloid cells were concentrated in fractions IV (39.2%) and V (33.5%), as can be seen in Table 1. The mean cellular composition of these fractions is also given in Table 1. Fraction IV contained 6.0% myeloblasts, 9.2% promyelocytes, and 33.5% myelocytes, whereas for fraction V the mean values were 9.3%, 20.7% and 31.0%, respectively (Table 1). More myeloblasts and promyelocytes, therefore, were concentrated in fraction V. Furthermore, only fraction V consistently showed a high percentage of immature myeloid cells (Table 2, experiments 1–6). In four other experiments this fraction was isolated a bit differently, as described in Materials and Methods. The percentages of immature myeloid cells in the latter fractions are also presented in Table 2 (experiments 7–10).

Figure 3 presents DNA histograms of the unprocessed bone marrow sample and of the pooled fractions in the lower region of the gradient (fraction V, Fig. 2). The high percentage of S-phase cells in fraction V is clearly demonstrated. Table 2 summarizes the results of DNA cytophotometry performed on the 10 isolated normal bone marrow samples. The mean percentage of S-phase cells was 25.4%, and G2 + M-phase cells 8.3%. The mean cell yield in these samples was 1.4 x 10⁶ (range 0.9–2.2 x 10⁶ cells).

**Leukemic Bone Marrow**

Leukemic bone marrow was obtained from five patients with full-blown acute myeloid leukemia. Their bone marrow contained 89.2% myeloblasts (range 85%–100%). The percentages of S-phase cells in these samples ranged from 4.1% to 15.0% (mean 9.7%).

**Density Gradient Centrifugation**

For the isolation of leukemic myeloblasts a Ficoll–Isopaque cushion was used with a density of 1.074 g/ml. After centrifugation an average of 65.2%
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Fig. 3. (A) DNA histogram of normal unprocessed bone marrow that contained 10.5% S-phase cells and 4.3% G₂ + M-phase cells. (B) DNA histogram of sample with a high percentage of immature myeloid cells (Fig. 2, fraction V); it contained 30.8% S-phase cells and 12.7% G₂ + M-phase cells. Channel numbers indicate an increasing amount of DNA. The large peak at channel 30 (2N DNA) indicates cells in G₁-phase, whereas the smaller peak at channel 60 represents cells with double the amount (4N) of DNA (G₂ + M-phase cells). Channels between the two peaks indicate cells in S-phase. Broken line is an electronic amplification (10-fold in A, 4-fold in B) of the information stored in the histogram below (solid line).

(range 54%-75%) of the applied myeloblasts was found at the interface. The interface fraction consisted of 93.6% (range 86%-100%) leukemic myeloblasts. The percentage of S-phase cells in these interface fractions ranged from 5.2% to 13.0% (mean 9.8%). This value was not different from the unprocessed bone marrow. This finding led to the conclusion that the leukemic cells recovered from the interface were representative of the whole bone marrow. For this reason Ficoll–Isopaque with a density of 1.074 was chosen. Lower densities resulted in a greater loss of cells at the interface. The results of the experiments
Fraction | I | II | III | IV | V
---|---|---|---|---|---
% S-phase cells | 2.6 | 1.6 | 3.8 | 7.9 | 25.1
% G2 + M-phase cells | 0.9 | 0.1 | 0.8 | 1.5 | 5.6

Fig. 4. Sedimentation (at 1 g) profile of leukemic bone marrow cells obtained after density gradient centrifugation. Appropriate fractions (I-V) were pooled and the percentages of S-phase and G2 + M-phase cells were determined by pulse cytophotometry. Fractions I-III contained leukemic cells hardly involved in DNA synthesis (G1- or G0-phase cells), whereas fraction V contained leukemic myeloblasts, of which 25.1% were in S-phase.

Velocity Sedimentation

When interface cells were applied to the gradient for velocity sedimentation at 1 g a separation could be obtained between cells involved in DNA synthesis and G1-phase cells. Figure 4 shows an example of such an experiment. The lower region (fraction V) of the gradient contained 25.1% S-phase cells, while the upper part (fraction I) held cells hardly involved in DNA synthesis. This separation was performed with interface cells from five patients with acute myeloid leukemia. Table 3 summarizes the results. In each case cell distribution profiles were obtained. These curves, however, were not identical to the one presented in Fig. 4. In several cases S-phase cells sedimented deeper in the gradient than fraction V depicted in Fig. 4. After each experiment fractions described here were similar to those obtained in a series of 10 experiments presented elsewhere. Hypotonic shock was not applied to leukemic cells because of the negligible number of erythroid precursor cells in these samples.

Table 3. Separation of G1- (or G0-) phase and S- and G2 + M-Phase Cells From Patients With Acute Myeloid Leukemia

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Upper Region</th>
<th>Lower Region</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S-Phase (%)</td>
<td>G2 + M-Phase (%)</td>
</tr>
<tr>
<td>1</td>
<td>3.0</td>
<td>1.0</td>
</tr>
<tr>
<td>2</td>
<td>3.3</td>
<td>0.1</td>
</tr>
<tr>
<td>3</td>
<td>2.6</td>
<td>0.9</td>
</tr>
<tr>
<td>4</td>
<td>4.0</td>
<td>1.0</td>
</tr>
<tr>
<td>5</td>
<td>2.5</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Interface cells, obtained after density gradient centrifugation of five leukemic bone marrow specimens, were subjected to velocity sedimentation as described in Materials and Methods. In each case cell distribution profiles were obtained. After each experiment fractions from the lower and upper region were pooled and processed for pulse cytophotometry and cytocentrifuge smears. The number of fractions pooled depended on the percentage of S-phase cells in the unprocessed bone marrow sample.
from the lower and upper regions were pooled and processed for pulse cyto-
photometry and cytocentrifuge smears. From the lower limb of the curve a
varying number of cells (1.0–3.0 × 10⁶ cells) were pooled. In order to obtain a
sample enriched with DNA-synthesizing cells, fewer fractions were pooled from
this area when the initial bone marrow specimen showed a low percentage of
S-phase cells. Contamination with lymphocytes in the upper region was 5.5%
(range 0%–11%), whereas the lower area of the gradient contained only leu-
kemic myeloblasts.

![DNA histogram](image)

Fig. 5. (A) DNA histogram of unprocessed leukemic bone marrow that contained 4.1% cells in S-phase and 0.5% cells in G₂ + M-phase. (B) DNA histogram of sample containing the proliferating leukemic cells (Fig. 4, fraction V); it contained 25.1% cells in S-phase and 5.6% in G₂ + M-phase. For explanation of the histogram see legend to Fig. 3. Broken lines represent a 10-fold electronic amplification of the solid lines.
DISCUSSION

During the maturation process from myeloblast to granulocyte the cell decreases in size and increases in density. Each cell type shows a Gaussian characteristic of density and size distribution, causing an overlap between the different cell types of the bone marrow. This result means that complete separation between mature and immature myeloid cells on the basis of density alone is not feasible.

Evans et al. reported the isolation of immature myeloid cells by centrifugation of normal bone marrow samples, after hypotonic shock, in a 15% Ficoll solution. After centrifugation the supernatant fraction contained 61%-84% immature myeloid cells. We repeated their experiments and found 63%-68% late immature myeloid cells, predominantly myelocytes. The percentages of S-phase cells in these samples were much lower compared to the samples obtained with the procedure described here. The latter contained considerably higher percentages of myeloblasts and promyelocytes than samples obtained by Evans' procedure.

Recently Bøyum reported the partial purification of immature myeloid cells from normal human bone marrow by centrifugation at room temperature on a standard Ficoll-Isopaque solution (density 1.077 g/ml). Samples were obtained with 51.0% ± 5.3% immature myeloid cells and a very low contamination with granulocytes (0.2%). In our hands this procedure (four experiments) yielded samples with 23%-31% immature myeloid cells and 4%-14% bands and polymorphonuclear leukocytes. In our opinion density gradient centrifugation alone did not result in samples qualitatively comparable to fractions obtained with the procedure described in this paper.

The disadvantage of our method is that only approximately 40% of the applied immature myeloid cells were recovered from the interface after density gradient centrifugation. However, all three steps described above were found necessary in order to obtain optimal separation. Pretreatment of the bone marrow sample on density gradients and with hypotonic shock (in order to remove erythrocytes, the majority of mature myeloid cells, and normoblasts) was required because only a limited number of cells could be applied to the gradient for velocity sedimentation. When more than 10^7 cells were applied considerable streaming occurred, resulting in a bad separation.

Leukemic bone marrow consists of a rather homogeneous population of cells. Density gradient centrifugation was performed only to remove the erythrocytes. The interface consisted of more than 90% leukemic myeloblasts. A similar observation has been made by Berrebi et al. DNA-synthesizing leukemic cells are large, whereas nonproliferating leukemic blasts are small. These cells can be separated by velocity sedimentation, as has been described above (Table 3). This method offers an opportunity to study separately G1-(or G0-) phase and S-phase cells from patients with acute myeloid leukemia. Similar separation techniques have been applied in AKR leukemia in mice with essentially the same results. The cell distribution profiles obtained for several patients with acute myeloid leukemia were not identical, indicating that the DNA-synthesizing cells from these patients do not sediment into the same region of the gradient. Apparently-DNA synthesizing cells from leukemic pa-
tients are different in size. In contrast to leukemic bone marrow, DNA-synthesizing myeloid cells from normal bone marrow were always present in the same region of the gradient. By application of more sophisticated methods\textsuperscript{22} it is perhaps possible to study cell cycle transfer in relation to cell size. If there is a gradual increase in size from early G\textsubscript{1}- to late G\textsubscript{2}-phase it should be possible to study phenomena occurring during these phases with a method based essentially on velocity sedimentation as outlined above.

It is now possible to study separately leukemic proliferative and nonproliferative cells. Furthermore, it is possible to compare rather pure normal immature myeloid cells with leukemic cells of the same proliferative capacity.

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Method for enrichment of proliferating myeloid cells from normal and leukemic human bone marrow

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