Leukocyte Density and Volume in Normal Subjects and in Patients With Acute Lymphoblastic Leukemia

By Alvin Zipursky, Eric Bow, Rama S. Seshadri, and Elizabeth J. Brown

A method is described for the separation of blood and bone marrow leukocytes on the basis of buoyant density, using a discontinuous Ficoll–Hypaque density gradient. The median cell densities of monocytes and lymphocytes were found to be 1.067–1.077 and 1.073–1.077 g/ml, respectively. The cells of the myeloid series were shown to increase in density with maturation; the myeloblasts had the lowest density (1.064–1.065 g/ml) and the neutrophils the highest (>1.080 g/ml). Cell volumes have been determined on isolated cell populations. The findings were: monocytes, 534 ± 47 cu μ; lymphocytes, 247 ± 18 cu μ; and neutrophils, 468 ± 24 cu μ.

Fourteen patients with acute lymphoblastic leukemia were studied. In four patients, the lymphoblasts were of low density (<1.068 g/ml), whereas the remaining patients had high density (>1.068 g/ml) lymphoblasts. These four patients had large numbers of lymphoblasts in the peripheral blood and a poor prognosis. Lymphoblast volumes were not different in these two groups and were unrelated to prognosis.

Many techniques have been described for the separation of blood and bone marrow leukocytes. This report describes a technique for the separation of bone marrow and peripheral blood cells on the basis of their buoyant density. We have applied this technique to the study of leukocyte density and volume in normal subjects and in patients with acute lymphoblastic leukemia.

MATERIALS AND METHODS

Fetal calf serum Krebs-Ringer medium (FCS-KR) was used either as a 20% or 30% solution (120 mM NaCl, 4 mM KCl, 1 mM MgSO₄, 0.016 mM phosphate buffer (pH 7.4), 0.25 mM CaCl₂, and 5.4 mM glucose).

Preparation of the Density Gradient

A 10%, solution of Hypaque was adjusted to an osmolality of approximately 294 mOsm (range = 285 300) as suggested by Shortman. The density of this solution varied from 1.055 g/ml to 1.062 g/ml. A 6%, Ficoll solution in 10%, Hypaque was prepared with a final osmolality of 294 mOsm (range = 291 305), a density of 1.074 to 1.090 g/ml, and a pH of 7.1 7.3.

In keeping with the findings of Williams et al., it was noted that the osmolality of the heavy-density medium could be maintained at a constant value when the Ficoll concentration of the medium was less than 10%. In some experiments, Isopaque-400 was used instead of Hypaque with no difference in results. Density was measured by determining the refractive index of the solution, and osmolality was determined by freezing-point depression.

Preparation of Cell Suspensions

The first step of the procedure consisted of a preliminary separation of the mononuclear cells and immature bone marrow elements from mature granulocytes and red blood cells. This pro-
procedure was adapted from that described by Boyum. A sample of heparinized (10 i.U./ml) blood or bone marrow was diluted in three volumes of sterile 0.15 M NaCl, mixed thoroughly and then carefully layered over a heavy-density gradient medium of 6%, Ficoll and 10%, Hypaque. This preparation was centrifuged at 400 g at room temperature for 40 min. The leukocytes found at the interface were removed and resuspended in 20%, FCS-KR and spun gently at 100 g for 10 min. The sedimented red cells and leukocytes were resuspended in an equal volume of the plasma NaCl supernatant. Two volumes of 3%, dextran in 0.15 M NaCl were mixed with one volume of the cell suspension. When the red cells had sedimented sufficiently, the leukocyte-rich supernatant was removed and the cells were washed twice in 20%, FCS-KR and resuspended in 30%, FCS-KR by centrifugation at 110 g for 10 min.

**Generation of the Density Gradient**

The washed interface cells were resuspended in 5 ml of the high density medium (1.080 g/ml) previously chilled at 4°C. The number of cells to be loaded onto the gradient was adjusted so as not to exceed 2 x 10^8 in order to be consistent with the recommendations of Shortman and Day. The usual load was in the range of 1-5 x 10^7 cells. Aliquots of this cell suspension in high-density medium were mixed with aliquots of the low-density medium (1.060 g/ml) to provide a range of densities. The discontinuous density gradient was generated with the assistance of a variable speed peristaltic pump by carefully layering these mixtures of Ficoll and Hypaque in decreasing order of density, one on top of the other, in a clear Nalgene 16 x 98-mm centrifuge tube (with an inner diameter of 13 mm). The speed of the pump was adjusted so as not to disturb the integrity of the interfaces between layers of differing densities. Ten layers of differing density were generated in this way.

The discontinuous Ficoll-Hypaque density gradient containing the cells was centrifuged at 4000 g in a swinging bucket (HB-4) rotor in a Sorval refrigerated centrifuge at 4°C. The cells came to equilibrium in the density gradient in 30 min, as confirmed in studies in which centrifugation was performed for 30 and 60 min with identical results for cell densities. The acceleration and deceleration were controlled manually over 10-min periods, as recommended by Shortman.

As a result of this centrifugation, the cells in the centrifuge tube were found to have distributed themselves at the interfaces of successive density steps in the gradient. The fractions were manually removed with siliconized Pasteur pipettes. Each fraction was approximately 1 ml, except for the first and last, which were 1.5 and 0.5 ml, respectively. The lines of force created by the centrifuge tended to direct the movements of the cells against the side of the centrifuge tube at the interfaces. Therefore, it was important to move the pipette in a slow circular motion around the edges of the interface while suctioning off the cells in order to maximize cell recovery. Each fraction thus recovered was delivered into a 15-ml conical Nalgene centrifuge tube for washing and analysis. Prior to washing, well-mixed samples were removed from each fraction for electronic cell counting and for refractive index measurement. Following this, the cells in each tube were washed and resuspended to a desired volume in 30%, FCS-KR. These suspensions were then ready for morphological studies and cell volume distribution analyses.

**Preparation of Cytocentrifuge Smears**

For preparation of cytocentrifuge smears, the cell concentration of the samples was adjusted so that an even monolayer of cells was obtained on the glass microscopic slides. The cell monolayers were stained with May-Grünwald Giemsa stain, and differential counts were performed.

Cytocentrifuge smears were used for differential cell counting because wedge smears of cell suspensions were found to provide an irregular distribution of cells, an observation made previously by others. Differential counts of mononuclear suspensions on centrifuge smears were compared to cell volume distributions (Coulter Counter) of these suspensions. In ten experiments, there were no significant differences in the differential counts obtained by the two methods (p > 0.20). It was concluded that a cytocentrifuge smear of a mononuclear suspension provided adequate samples for differential counting.
Analysis of Cell Densities

For each fraction, the number of a given cell type was expressed as a percentage of the total numbers of that cell in all fractions. The cumulative frequencies of each cell type was recorded on probability paper for determination of median density.

Cell Volume Analysis

Leukocyte volume was determined electronically by pulse height analysis using an electronic cell counter (Coulter Counter, Model B, Coulter Electronics, Mississauga, Ont.) in combination with a 64-channel pulse height analyzer (Coulter Model P64) and an X Y plotter. The transducer aperture was 100 μ in diameter and 75 80 μ in length. The sample of cells, prepared as described above, was suspended in 30°, FCS KR and was diluted in Coulter Isoton such that the cell count was less than 50,000/cu mm. The sample was then counted by the machine, and the volume distribution was plotted automatically. When the suspension contained one cell type, a single distribution curve was found from which the modal volume was determined. In mixtures of cells with overlapping cell volume, the analysis became somewhat more complex. Usually, the modal volumes could be determined by eye and the extremities of the distribution curves could be determined by arbitrarily completing the two curves. For more accurate analysis of overlapping cell volume distributions, the data were plotted as a cumulative frequency on probability paper.

Hematologic Techniques

Blood and bone marrow smears were stained by May-Grünwald Giemsa and by the following special stains: peroxidase, PAS, Sudan Black B, as described with modification;10 12 chloroacetate esterase and nonspecific esterase by the technique of Yam et al.13 The PAS reaction was assessed by counting all the PAS positive lymphoblasts (containing either granules or “chunks”) and expressing them as a percentage of total lymphoblasts.

Patients

Fourteen patients with acute lymphoblastic leukemia (ALL) were studied (Table 1). Ten patients were studied before treatment, at the time of initial presentation, and four (patients 1,7,12,13) at first hematologic relapse.

The morphological diagnosis of ALL was made from bone marrow aspirates in 11 patients and from peripheral blood examination in three patients. The diagnosis of ALL was made on the basis of the following criteria concerning the leukemic leukocyte: (1) characteristic morphology

<table>
<thead>
<tr>
<th>Case</th>
<th>Age</th>
<th>Lymphoblasts in Blood x 10^9 liter</th>
<th>Median Lymphoblast Density (g/ml)</th>
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<tr>
<td>1</td>
<td>3</td>
<td>0</td>
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<tr>
<td>2</td>
<td>6</td>
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<tr>
<td>14</td>
<td>31</td>
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showing cells with high nuclear-cytoplasmic ratio and indistinct nucleoli; (2) positive PAS reaction with "chunk" positivity; (3) negative peroxidase reaction and Sudan Black B stain; and (4) negative stain for chloroacetate esterase and nonspecific esterase.

Remission was induced with vincristine and prednisone in ten; vincristine, daunorubicin, and prednisone in two; and the two adult patients (13 and 14) received daunorubicin, cytosine arabinoside, vincristine, and prednisone. All patients, except patient 1, received central nervous system (CNS) prophylaxis consisting of cranial irradiation (2400 rads) and five doses of intrathecal methotrexate. Patients 12 and 13 had CNS leukemia before initiation of prophylaxis. Patient 13 was the only case with a mediastinal mass. Hepatosplenomegaly was absent in patients 1, 2, 4, and 5.

RESULTS

Preliminary Separation

The separation was carried out by layering a cell suspension over a Ficoll-Hypaque solution having a density of approximately 1.080 g/ml. In 26 experiments with normal blood, the interface was found to contain mostly lymphocytes, with most of the remainder being monocytes (23% ± 11%). The contaminating cells were evaluated in seven experiments and were found to include basophils (3.5% ± 2.9%) and neutrophils (1.1% ± 1%). The cell button in these experiments contained predominately neutrophils (89.5% ± 6.6%), with 0.6% (SD = ±0.8%) lymphocytes and monocytes and 9.9% (SD = ±6.4%) eosinophils.

![Fig. 1. Photomicrographs of cytocentrifuged preparations of human bone marrow cells recovered from various fractions of the Ficoll-Hypaque density gradient. (A) Myeloblasts from light-density fraction (density: 1.063 g/ml). (B) Promyelocytes recovered from intermediate-density fraction (density: 1.068 g/ml). (C) Myelocytes recovered from intermediate-density fraction (density: 1.075 g/ml). (D) Heavy-density fraction containing metamyelocytes, bands, a segmented neutrophil, lymphocytes, and erythrocytes (density: 1.079 g/ml).]
LEUKOCYTE DENSITY AND VOLUME IN ALL

Cell Separation in the Density Gradient

As described in Materials and Methods, the interface cells obtained by preliminary separation were placed on the gradient. Cell recovery from this gradient was calculated in eight experiments and was found to be 67\% (SD = ±14\%) of interface cells added. In Fig. 1, there is a series of photomicrographs demonstrating the microscopic appearance of human bone marrow cells recovered from various fractions of the gradient in a typical experiment. Figure 2 shows the distribution of cells of the myeloid series separated according to maturity, with the more immature cells in the lighter density regions. The results of four such separations are shown in Table 2. Neutrophils were not included in these results, since the vast majority of these cells sedimented out in the preliminary separation into the cell button. Since the density of the medium in the preliminary experiment was as heavy as, or heavier than, any of the gradient, it followed that neutrophils had the highest density of any cell in the myeloid series and that in each subject studied there was a progressive increase in median cell density during myeloid maturation.

Cell Volume Analysis

Suspensions of lymphocytes and monocytes were obtained by the preliminary separation technique from normal blood. The modal volumes of human peripheral blood lymphocytes and the monocytes in a series of 26 experiments

<table>
<thead>
<tr>
<th>Table 2. Median Densities* of Normal Bone Marrow Cells Separated on a Discontinuous-Density Ficoll-Hypaque Gradient</th>
</tr>
</thead>
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<td>Cell Type</td>
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</tr>
<tr>
<td>Promyelocyte</td>
</tr>
<tr>
<td>Myelocyte</td>
</tr>
<tr>
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</tr>
<tr>
<td>Lymphocyte</td>
</tr>
<tr>
<td>Monocyte</td>
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</table>

*Density is expressed as g/ml.
were found to be 247 cu μ (SD = ±18) and 534 cu μ (SD = ±47), respectively. When lymphocyte volumes were determined in the fractions removed from the discontinuous density gradient, there appeared to be a difference in the cell volume of lymphocytes obtained from each fraction. In each of five experiments, lymphocyte volumes fell progressively from the lowest density to the highest density. Data from 26 experiments showed an average modal volume of normal peripheral blood granulocytes of 468 cu μ (SD = ±24).

Density Distribution of Lymphoblasts

Figure 3 shows the density distribution of lymphoblasts of 14 patients. In nine patients, the lymphoblasts were distributed mainly in the high density region, the peak values of densities ranging from 1.074 to 1.080 g/ml. In four patients, the lymphoblasts were distributed in the low-density region, the peak values of densities ranging from 1.060 to 1.065 g/ml. Lymphoblasts of patient 8 were distributed in the intermediate range. For the purpose of further analysis of data, the patients were divided arbitrarily on the basis of median lymphoblast density into “high-density” (>1.068 g/ml) and “low-density” (<1.068 g/ml) groups (Table 1). Most of the samples were bone marrow, although two of the four low-density group and one of the high-density group were peripheral blood. In patient 12, median lymphoblast density was determined from peripheral blood and bone marrow samples with similar results (blood 1.060 g/ml, bone marrow 1.063 g/ml).

Fig. 3. Lymphoblast density in 14 patients with acute lymphoblastic leukemia. The number of lymphoblasts per fraction of a discontinuous density-gradient separation are plotted on the ordinate. The lymphoblasts in each fraction are expressed as a percentage of the total number of lymphoblasts recovered. The numbers (1–14) indicate the case number. The continuous lines represent the high-density group. The broken lines show the low-density group.
Fig. 4. The modal volume of isolated lymphoblasts in low-density (<1.068 g/ml) and high-density (>1.068 g/ml) groups.

**Lymphoblast Density and Clinical Features of Leukemia**

The patients with low-density lymphoblasts had initial lymphoblast counts in the peripheral blood (Table 1) ranging from $60 \times 10^9$/liter to $429 \times 10^9$/liter. In the high-density group, only two patients had values in this range and five cases had no lymphoblasts in the peripheral blood.

In the low-density lymphoblast group, the percentage of PAS-positive lymphoblasts ranged from 1.6% to 72%; in the high-density group, it ranged from 1.5% to 93%. Both groups of patients had "chunk" positive lymphoblasts. Figure 4 shows the relation between modal lymphoblast volume and the two density groups. Each group included some patients with large and others with small lymphoblasts.

In the low-density group, two (patients 12 and 13) of the four patients developed CNS leukemia before CNS prophylactic therapy. All four patients suffered hematologic relapse within 11 mo of diagnosis. Three of them died 5, 6, and 10 mo after diagnosis.

In the high-density group, all patients achieved initial remission within 6 wk of treatment. Patient 1 developed CNS leukemia 38 mo after diagnosis and died 2 mo later. This patient did not receive CNS prophylaxis at the time of presentation. Three patients (3, 5, and 7) had hematologic relapse 10, 13, and 16 mo after diagnosis. The other six were in complete remission 8-32 mo after diagnosis.

**DISCUSSION**

In this study, we have described a technique for the separation of the nucleated cells of blood and bone marrow on the basis of cell density. The technical quality of the cytocentrifuge smears in all instances was excellent, so that morphological assessment and identification of cell types obtained from the density gradient could be performed readily. Dye exclusion (Trypan Blue) studies have been done after preliminary separation, and in all instances over 95% of the cells excluded dye.

An important feature of this method was that the osmolality of the separation medium remained constant with changes in density. A change in the osmolality of the gradient medium may cause a change in cell density, resulting
in failure of proper cell separation. In our technique, the concentration of the Ficoll was maintained at approximately 6%, since Ficoll in concentrations above 10% causes a nonlinear rise in osmolality. For this reason, studies using Ficoll in concentrations of 14%-25% are difficult to interpret, and cannot be compared to our observations.

The recovery of cells from this discontinuous Ficoll-Hypaque density gradient system was 67.7% (SD = ±14%). Cell recovery techniques described by other investigators using a variety of density gradient systems for leukocyte separation have recoveries of 50%-90%.

Several features of the technique were developed in order to minimize the problems associated with a discontinuous gradient. In keeping with Shortman's recommendations, the cell samples were mixed with a high-density solution and incorporated throughout the gradients, so that there was a gradient of cells oriented in the tube in the same direction as the density gradient. In this way, the problems associated with cell clumping and interface impedance were reduced. The mechanical disturbances of swirling and mixing were minimized by slow acceleration and deceleration during centrifugation. The temperature during centrifugation was maintained at 4°C to preserve the cells and to reduce thermal convection effects. Close attention to these details of procedure resulted in a system for cell fractionation with a readily reproducible pattern of separation.

The distribution of cells among the fractions of the gradient suggested a Gaussian distribution for each cell type. Although not specifically reported, inspection of the density distribution curves presented in several other studies also suggested Gaussian characteristics of density distribution.

The density of cells in the myeloid series increased with maturity. This observation was consistent with those of others, although median densities were not given in those studies. It is apparent, however, from review of these published data that the densities of cell types in their studies were similar to ours. It was difficult to assign, from our data (Table 2), median densities for each specific cell type that were consistent in all experiments. This result may be related to a real difference between patients or simply to experimental variation. In either event, each experiment displayed a consistent pattern of increasing density, with maturation within the myeloid series (Table 2, Figs. 1 and 2). The low density of the youngest cells of the myeloid series is consistent with the observations of others.

In 1973 Moore et al. reported a range of density for monocytes of 1.063-1.078 g/ml, based upon experiments using continuous albumin density gradients. These values were quite similar to those reported here, namely 1.061-1.077 g/ml. The relatively wide range in density for this cell, along with the changes in some individuals from time to time, suggests that monocytes may vary in density, either physiologically or as a result of disease.

Lymphocytes were found throughout the entire gradient. It seems likely that this reflects the heterogeneity of this population and, in fact, it has been demonstrated that B cells have distinctly low density. The separation of cells by our technique permits the determination of volume of specific cell types. Others have used membrane-lysing agents to remove
unwanted red cells for their leukocyte volume measurements.\textsuperscript{28-30} It is known that such agents damage leukocyte membranes, as well as red cell membranes.\textsuperscript{31} Furthermore, we have also observed that saponin causes leukocytes to shrink and, therefore, to be sized as though of smaller volume. This effect might explain why modal volumes in some studies using saponin\textsuperscript{29} were less than those reported by others.

Few studies have attempted cell volume analyses after achieving relatively pure populations of cell types, using a definitive cell separation procedure. Westring et al.\textsuperscript{32} separated lymphocytes from peripheral blood on glass bead columns. Zucker and Cassen\textsuperscript{33} separated peripheral blood cells on continuous albumin density gradients. Lymphocyte volumes reported in these two papers were similar to those observed in the present study. Furthermore, Zucker and Cassen\textsuperscript{33} reported that lymphocyte volume tended to relate inversely to density, an observation we have been able to confirm.

Applying this technique to ALL, we observed that in the majority of cases (Fig. 3) the density of the lymphoblast was high, compared to the myeloblast and to most lymphocytes. In addition, it was evident that four patients had a distinctly lower cell density than the remainder (Fig. 3, Table 1). The lymphoblasts in these cases did not differ from those in the high density group in terms of morphology, PAS staining, or cell volume (Fig. 4).

All four of the cases with low-density lymphoblasts were characterized by sudden onset of disease, high leukocyte counts, and a short duration of remission after diagnosis. The patients in this group had marked organomegaly and, as seen in Table 1, all had lymphoblast counts at diagnosis in excess of $50 \times 10^9/$liter, whereas this occurred in only two of the high-density group.

It has been reported that a poor prognosis is found in those cases of ALL in which the lymphoblasts stain weakly with PAS\textsuperscript{34} or are of large volume.\textsuperscript{35} We found no such relationships in keeping with the negative findings of others.\textsuperscript{36,37} In our series, the only distinctive property of the lymphoblasts in those cases with the poorest prognosis was their low density.

**ACKNOWLEDGMENT**

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