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Light Scatter Characteristics of Erythroid Precursor Cells Studied in Flow Analysis (40318)

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Flow analysis is a powerful new tool to study characteristics of individual cells in suspension (1). The presently available flow instruments measure either scatter of monochromatic light by cells or fluorescence of fluorochrome-labeled cell structures. In addition, light scatter (LS) or fluorescence can be a discriminating parameter by which selected cells can be separated electronically for additional study.

Bone marrow is a complex mixture of cells of several developing myeloid lines (granulocytes, erythrocytes, megakaryocytes, and monocytes) as well as lymphocytes. Effective study of these cell types requires samples highly enriched with an individual cell type. For the flow instruments to sort a cell type, selection based on either LS or fluorescence must be made. Fluorescence usually requires irreversible alteration and killing of cells. Since LS study is cell-sparing, sorted cells would be chemically unaltered and possibly biologically active. Studies of bone marrow cells in flow analysis have shown the possibilities of separating selected cell types of study (2, 3). In previous reports from this laboratory (3), emphasis has been placed on separating granulocyte precursors of varying stages of development. In this report, the feasibility of separating erythrocytes and erythroid precursors in relatively pure form is addressed. This was accomplished by a preliminary isopycnic fractionation of rabbit marrow on density gradients followed by flow analysis of the gradient fractions, using LS profiles to determine sorting parameters.

Materials and methods. Preparation of marrow suspensions. Rabbit marrow was removed from the long bones, filtered, and washed as previously described (4) except that hypotonic lysis of erythroid precursors was not included.

Density gradient fractionation of bone marrow. Washed bone marrow cells were suspended in Ficoll/Hypaque solution and dispersed in a linear density gradient formed with the Beckman gradient former. The mixing solutions had densities of 1.0478 and 1.1579 g/ml. Gradients were formed in 13-ml tubes for the SW41 rotor and the cells were separated isopycnically during a 40-min centrifugation at 4300*g*.

Flow analysis and electronic cell sorting. Fractions from the preliminary isopycnic separation of cells were analyzed with a Coulter Electronics Company TPS-1 sorter. Cells were analyzed at a flow rate of 1000 to 3000 cells per second and LS histograms were generated as described previously (3). The distinct and reproducible distributions in the LS histograms were used to set electronic sort windows by which 100,000 cells were sorted in each of two windows simultaneously.

Sorted cells were collected in fetal calf serum and collected on microscope slides in a Shandon cytocentrifuge. Differential cell counts were performed after staining with Wright's stain.

Results. Light scatter profiles of bone marrow cells at differing buoyant densities. Blood cell precursors of the bone marrow, both erythroid and myeloid, increase in buoyant density as they mature. Thus a preliminary separation of bone marrow cells by isopycnic sedimentation in Ficoll/Hypaque gradients allows collection of gradient fractions near the top of the gradient which are rich in immature cells and fractions of increasing maturity progressing to the bottom of the gradient (4).

The cells recovered from each density gradient fraction (I–X) were subjected to flow analysis. The LS histograms, with the cell numbers on the ordinate and increasing LS intensity on the abscissa, are shown in Fig. 1. For clarity, 4 of the 10 gradient fractions which best illustrate the typical changes in the profiles from top to bottom of the gra-

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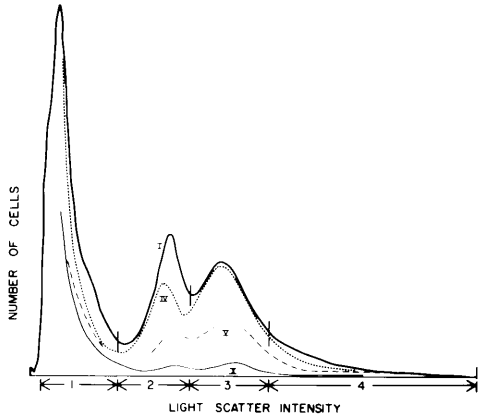

FIG. 1. Light scatter (LS) histograms of rabbit marrow cells. Cell number is plotted on the ordinate and increasing LS is indicated on the abscissa. Arabic numerals refer to limits of windows for electronic sorting of cells. Roman numerals refer to LS patterns of fractions derived from preliminary density gradient fractionation of the marrow (fraction I at top, fraction X at bottom of the density gradient).

dients are shown. The Arabic numerals on the abscissa designate sort fractions and indicate the segments under the LS profile chosen for individual electronic sorts. In gradient fraction X, too few cells were available for a fourth sort fraction.

The pattern consists of distributions representing distinct classes of cells with similar LS properties. The peak included in sort fraction 1 dominates in terms of cell number. Each curve is adjusted to show the peak at the top of the histogram so that the relative proportions of the sort fractions can be compared.

In gradient fraction I (top of the density gradient) the peak included in sort fraction 2 is more prominent than the third peak (sort fraction 3), but both peaks are similar in gradient fractions closer to the bottom of the density gradient, and both are small compared to the peak in sort fraction 1 at the bottom of the gradient.

In terms of total cells, 46% of the cells in gradient fraction I are erythroid, over half of which are nucleated. Erythrocytes make up 84% of the cells of the gradient fraction X, but only 3% of these are nucleated.

The differential counts of individual sort fractions in Fig. 2 show the distribution of cell classes in several sort fractions derived from each density gradient fraction. The differential cell counts from each sort fraction are reported in three categories, represented by the three bars under each sort designation in Fig. 2. The "lymph" bar indicates lymphocytes and smudged nuclei (shaded portion), which may sort with lymphocytes. The "RBC" bar indicates erythrocytes and erythroid precursors. The latter are indicated by shading and "nRBC". The third bar ("gran") in each sort indicates granulocytes, mature polys, and their precursors.

Sort 1 is predominantly an erythroid fraction, sort 2 is enriched with nucleated red cells, and sorts 3 and 4 are primarily granulocyte fractions. If sort fraction 2 of gradient IV (mid-gradient) is chosen, a sample of cells is obtained which is over 90% erythroid, 64% of the cells being nucleated erythroid precursors.

An example of this fraction is shown in Fig. 3. This photomicrograph shows a group of nucleated erythrocyte precursors and one larger cell which may be lymphoid. The granulocytes chiefly responsible for the LS peak in sort fraction 3 are more mature than those which predominate in sort fraction 4. The larger less mature granulocytes scatter more light than the more mature cells (3).

Discussion. The scatter by cells of an incident beam of light is determined in part by the size of the cell, but also depends on reflection from cell surfaces, phase-shift in light passing around or through the cell, and diffraction of light by internal structures within the cell. The instrument involved in this study utilizes a light detector which collects light scattered 2° to 20° from the incident beam. It is known that cell size is the most important determinant at low angles of scatter (2° to 5°) and presumably internal structure plays a greater role in determining the intensity of scatter at larger angles (5).

It is clear from these studies that cell size is not the only determinant of scatter. The mature erythrocytes and reticulocytes scatter less light than any other cell type in the marrow, and there is a distinct separation of peaks of nucleated and non-nucleated erythrocytes. Since the size of the maturing red cell precursors decreases in a continuous fashion, if size were the major determinant of LS, there would be one broad, continuous peak

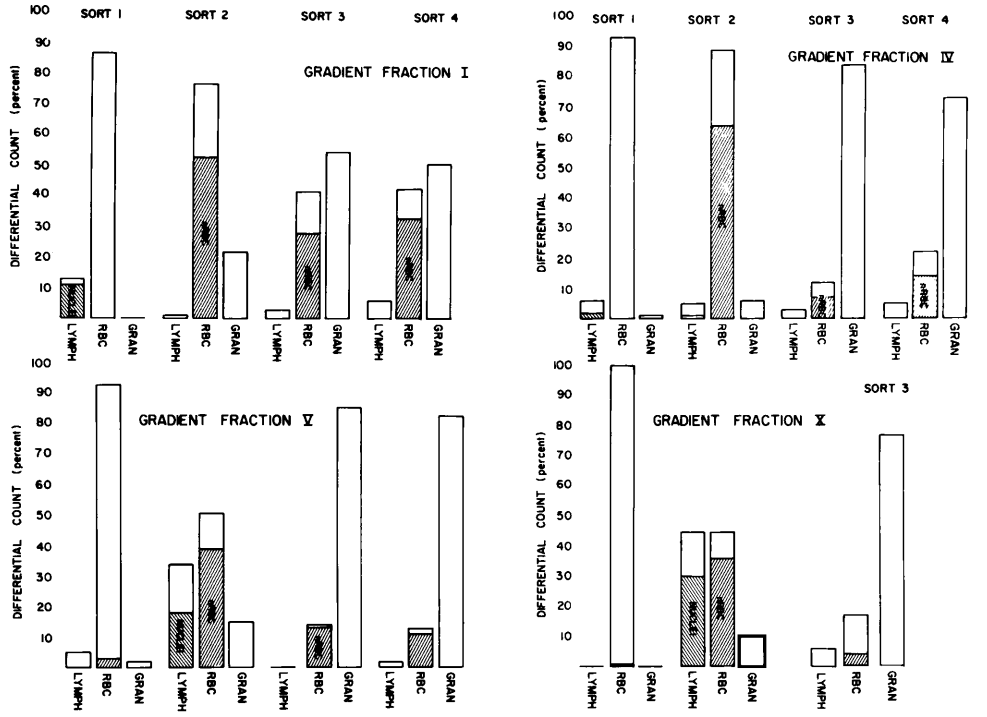

FIG. 2. Differential cell counts of cells sorted from density gradient fractions whose LS profiles are seen in Fig. 1. Each sort fraction contains cells obtained from the abscissa in Fig. 1. Each sort fraction is reported as lymphs (lymphocytes and smudged nuclei), RBC (erythrocytes and nuclear RBC), and gran (neutrophils and their precursors). In gradient fraction X only three sort fractions were collected.

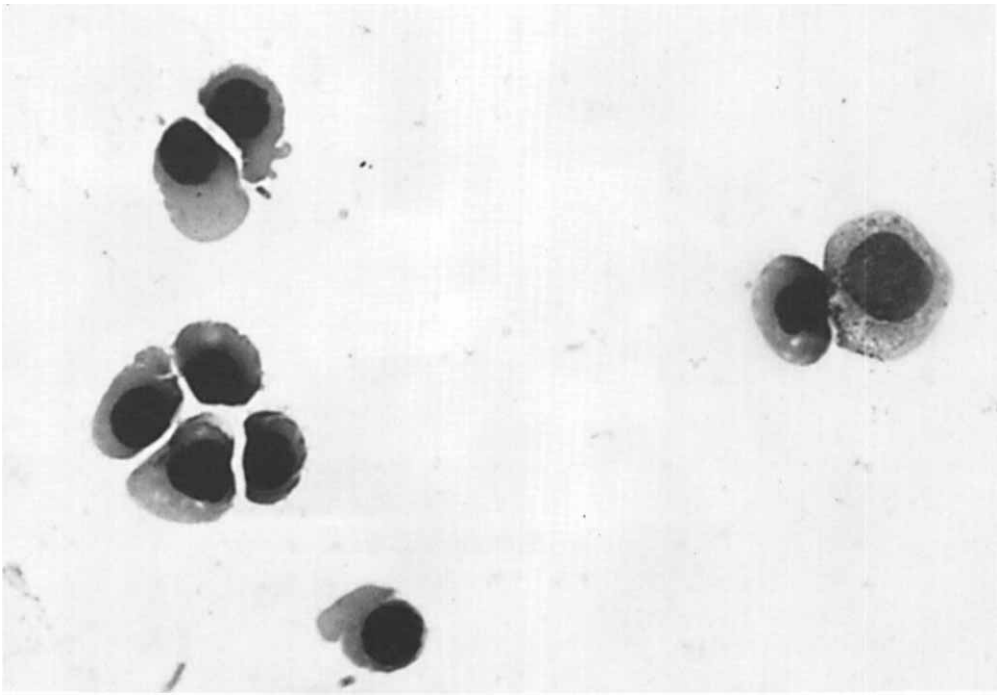

FIG. 3. Photomicrograph of sort fraction 2 of density gradient fraction IV. Cells shown are nucleated erythrocyte precursors and one probable lymphoid cell. Original magnification, × 1000.

of erythroid LS. Instead, discrete distributions were observed (Fig. 1). This was also evident in other LS studies from our laboratory (3) in which it was shown that lymphocytes of various sizes were found to have very similar LS properties. It is evident that the character of the nucleus is an important LS determinant.

Granulocytic cells tend to scatter more light than erythrocytes, normoblasts, or lymphocytes. This is due no doubt to both greater cell size and much greater complexity of cytoplasmic organelles.

It is evident that LS of cells, especially when combined with separation based on buoyant density differences, is a useful means of isolating erythrocyte precursors for study.

Summary. Light scatter (LS) differences among cells of rabbit marrow was studied by flow analysis using a Coulter two-parameter cell sorter. A preliminary fractionation of the marrow into samples enriched with cells of varying degrees of maturation was accomplished in Ficoll/Hypaque density gradients. Subsequent study of each of these cell samples in flow analysis demonstrated unique LS profiles which distinguished erythrocytes from nucleated erythroid precursors and granulocyte precursors. The combined separation procedures made it possible to sort fractions of erythroid precursors with as high as 90% erythroid cells, two-thirds of which were nucleated precursors.

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