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Identification of Discrete Classes of Normal Human Peripheral Lymphocytes by Multiparameter Flow Analysis (38246)

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(Introduced by P. M. Kraemer)

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The application of the metachromatic fluorochrome acridine orange to vitally stain human leukocytes is providing new information in blood cell studies. For example, Jackson (1) demonstrated that acridine orange-stained leukocytes exhibit a uniform green fluorescent nucleus with red fluorescent cytoplasmic granules. He was able to microscopically distinguish the different leukocytes on these bases. Methods of fluorescence microspectrophotometry have been employed by West (2) to study the metachromatic staining properties of cells, including leukocytes. Acridine orange-stained leukocytes have been machine-classified by Adams and Kamentsky (3) into three classes (i.e., lymphocytes, monocytes, and granulocytes) on the basis of red fluorescence measurement of cytoplasmic granulation. We have verified their results by sorting leukocytes according to red fluorescence measurement (4). Melamed *et al.* (5) have recently examined the feasibility of studying differential leukocyte counting using automated machine analysis from leukemic patients undergoing chemotherapy.

We report here the separation of normal human lymphocytes into two distinct groups based on green-to-red fluorescence ratio measurement of acridine orange-stained leukocytes using improved multiparameter cell analysis and sorting instrumentation (6). Using this methodology for analysis of nuclear-to-cytoplasmic staining properties, leukocytes were sorted and microscopically identified as granulocytes, monocytes, and two types of morphologically different lymphocytes. Leukocyte fractions sorted on the basis of green/red fluorescence ratios and subjected to cell volume analysis exhibited somewhat different but overlapping size distributions. Green/red fluorescence ratio distributions of leukocytes from three normal human donors showed quantitative variation of lymphocytes within the two subpopulations. Two parameter fluorescence analysis of stained leukocytes also distinctly indicates four leukocyte classes. These results suggest that the sorted lymphocyte groups consist of the two cell types normally classified as large and small lymphocytes.

Materials and Methods. Fresh whole peripheral blood from 12 normal human donors was collected via venipuncture in 0.01 ml of 10% EDTA/ml of blood and chilled at 4° for 5 min prior to staining for fluorescence analysis and subsequent cell sorting. Leukocytes were stained and prepared for analysis by diluting one part whole blood with 25 parts of acridine orange stain solution (1 μg of acridine orange/ml of 0.85 M NaCl buffered with 0.005 M phosphate, pH 7.4) (3). Stained leukocytes examined with the fluorescence microscope exhibited a relatively uniform green nucleus with distinct red cytoplasmic granules. Cytoplasmic granules of granulocytes were large and coarse, compared to finer dust-like particles within lymphocytes. Since erythrocytes do not take up perceptible amounts of acridine orange, hemolysis was not required. Blood samples were maintained on ice during analysis to stabilize staining dynamics.

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A comprehensive description of the instrumentation utilized in this study has been presented elsewhere (6). The blood cell suspension enters a flow chamber where electrical and optical sensors measure their physical and biochemical cellular properties (Fig. 1). Cells first pass through a cell-volume sensing orifice (Coulter principle) and then intersect an argon laser beam (488 nm wavelength), causing fluorescence. Two-color green (520–580 nm) and red (590–800 nm) fluorescence is electro-optically measured using a dual photomultiplier tube array. After flourescence measurement, the liquid stream carrying the suspended cells emerges into air and is broken into uniform liquid droplets (45,000/sec) by use of a piezoelectric transducer. Thus, blood cells were isolated into liquid droplets with approximately 1% of the droplets containing leukocytes. Erythrocytes, which outnumber leukocytes approximately 1000:1, were contained in all droplets.

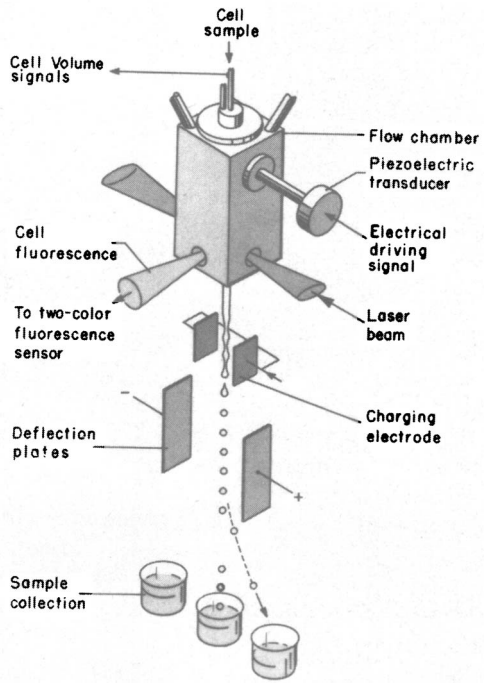

FIG. 1. Multiparameter cell separator illustrating the flow chamber, laser illumination, droplet generation, charging and deflection scheme, and sample collection.

Fluorescence sensor signals were electronically processed on a cell-by-cell basis as single parameters and ratios and were displayed as pulse-amplitude frequency distribution histograms using a multichannel pulse-height analyzer. Two parameter analysis was accomplished by selecting single and ratio fluorescence signals as inputs to a dual parameter pulse-height analyzer where three-dimensional frequency distribution histograms and two-dimensional contour diagrams were displayed. Processed fluorescence ratio signals were used to activate cell sorting if the amplitudes fell within adjustable preselected ranges. An electronic delay was then triggered on, permitting adequate time to elapse for the leukocyte being sorted to reach the droplet formation point. A group of droplets containing the selected leukocyte were then electrically charged and electrostatically deflected into collection beakers containing 1–2 ml of phosphate-buffered saline with 5% fetal calf serum. Those leukocytes not satisfying the sorting criteria passed undeflected into a separate vessel. Since erythrocytes were contained in all droplets, they were sorted along with selected leukocytes. The sorted suspension was introduced into a cytocentrifuge[1] and deposited onto a microscope slide for fixation and counterstaining. Sorted leukocyte samples were fixed in absolute methanol (12 hr), counterstained with Wright's Giemsa, and classified microscopically.

Leukocytes were also sorted into 5 ml of phosphate-buffered saline and then reintroduced into the cell sorter for volume distribution analysis. Leukocyte volume distributions were obtained without hemolysis of associated erythrocytes by analyzing only those volume signals from cells having green fluorescence signals. Erythrocyte volume distributions were similarly obtained by recording the volume signals of those cells lacking green fluorescence.

Results. Green and red fluorescence pulse-amplitude distributions of normal human leukocytes vitally stained with

[1] Shandon Scientific Co., Sewickley, PA.

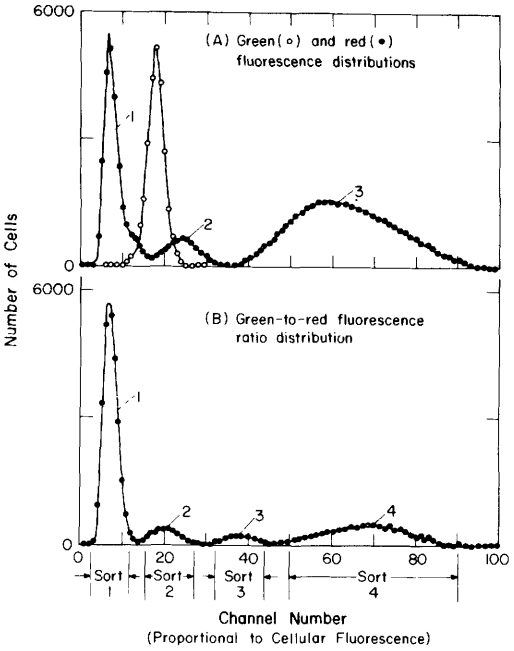

FIG. 2. Frequency distribution histograms of acridine orange-stained normal human leukocytes: (A) green and red fluorescence distributions, and (B) green-to-red fluorescence ratio distribution showing selected cell sorting regions.

acridine orange are shown in Fig. 2A. The green fluorescence distribution is unimodal, illustrating similarities in leukocyte nuclear staining, whereas the red fluorescence distribution shows three distinct peaks which characterize human leukocytes into three groups on the basis of cytoplasmic granules which fluoresce red. Peaks 1, 2, and 3 have been reported (3) to consist primarily of lymphocytes, monocytes, and granulocytes, respectively. Sorting of human leukocytes on the basis of red fluorescence has confirmed these results (4). The green/red fluorescence ratio pulse-amplitude distribution (Fig. 2B) shows four distinct leukocyte groups (peaks 1–4), permitting leukocyte characterization as a function of nuclear-to-cytoplasmic staining properties. Leukocytes having green/red fluorescence ratio signal amplitudes corresponding to sort regions 1, 2, 3, and 4 were separated, counterstained, and microscopically identified as granulocytes, monocytes, and two types of lymphocytes, respectively. The small shoulder located on the right side of the lymphocyte peak of the red fluorescence distribution (Fig. 2A) accounts for peak 3 of the green/red fluorescence ratio distribu-

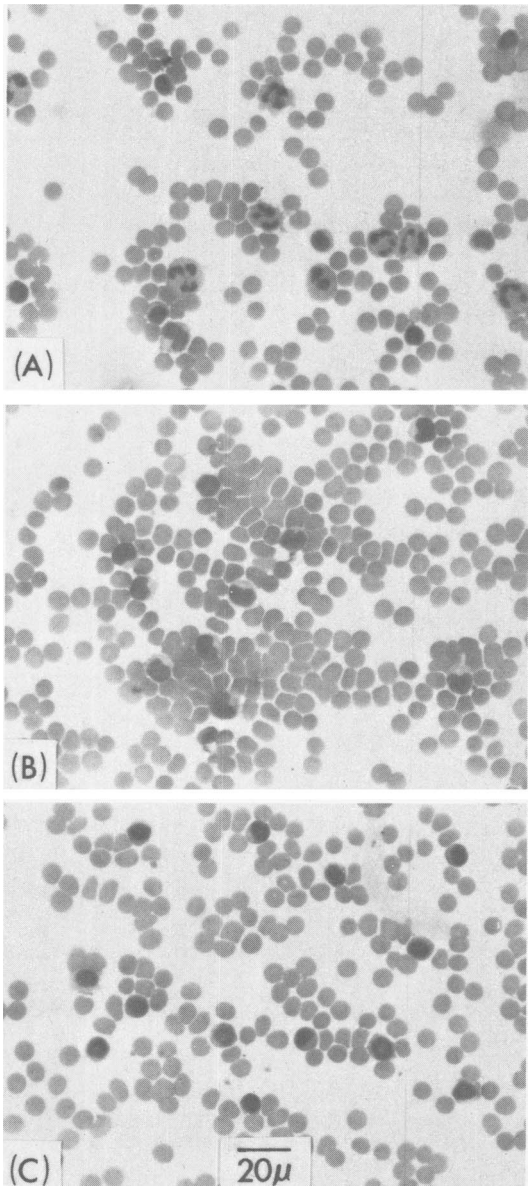

FIG. 3. Photomicrographs of sorted normal human leukocytes counterstained with Wright's Giemsa: (A) enriched leukocyte sort; (B) sorted large lymphocytes; and (C) sorted small lymphocytes. Erythrocytes contained in the photomicrographs were separated along with selected leukocytes.

tion. This is verified using two parameter analysis methods as described below.

Figure 3A shows a photomicrograph of an enriched leukocyte population obtained by sorting leukocytes based on green fluorescence signals between channels 5–30 of Fig. 2A. Differential cell counts made on blood smears are in close agreement with the enriched leukocyte sort (4). Similar results also are obtainable by sorting leukocytes based on green/red fluorescence ratios between channels 3–100 of Fig. 2B. Figures 3B and 3C show photomicrographs of sorted and counterstained lymphocyte fractions corresponding to sort regions 3 and 4 of Fig. 2B, respectively. Microscopic examination indicates that fraction 3 is principally large lymphocytes and fraction 4 small lymphocytes. This observation was further validated by subsequent cell volume analysis of the sorted leukocyte fractions. The nuclear chromatin of the small lymphocytes counterstains more densely than the chromatin of the large lymphocytes, indicating a varied degree of nuclear chromatin condensation between these cell types. Also, the nucleus of the large lymphocytes is kidney-shaped, whereas the nucleus of the small lymphocytes is round. These results imply two types of lymphocytes which are morphologically and functionally distinct. Leukocytes corresponding to sort regions 1 and 2 (Fig. 2B) were identified microscopically as granulocytes (principally neutrophils) and monocytes, respectively, and were identical to those previously sorted on the basis of red fluorescence measurements (4).

Figure 4A shows a typical volume distribution of sorted leukocytes which compares favorably with that reported by others (7). This distribution was obtained by sorting leukocytes having green fluorescence signal amplitudes between channels 5–30 of Fig. 2A, reanalyzing the sorted cells, and recording the total leukocyte volume spectrum. Leukocytes contained in peak 1 are primarily lymphocytes, while those within peak 2 are principally neutrophils (7). Volume distributions of leukocytes from various normal human donors show some quantitative variations within the two principal peaks. The volume distribution of sorted erythrocytes was used as a marker to normalize the relative size determinations. Individual cell volume distributions of leukocytes sorted according to sort regions 1–4 of Fig. 2B are shown in Fig. 4B. Sorted fractions 3 and 4 consist of 2 different lymphocyte size classes having somewhat overlapping volumes. Minor shifts in degree of overlap of the two lymphocyte volume distributions were observed within different individuals. Sorted leukocyte fractions 1 and 2, which were identified as granulocytes and monocytes, are characterized by considerably larger and overlapping volumes.

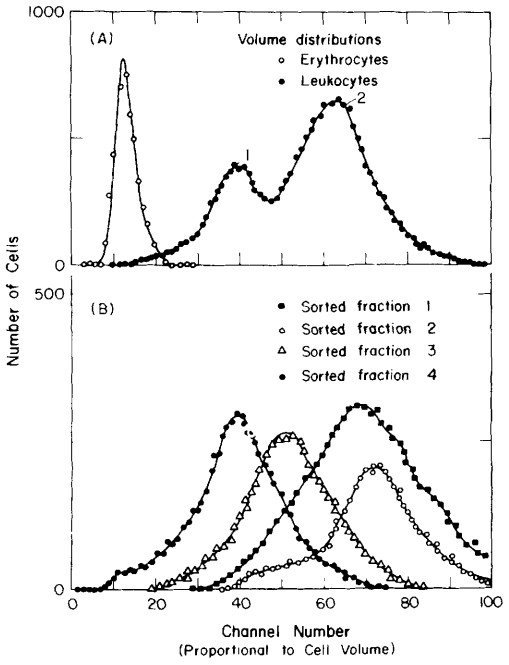

FIG. 4. Volume distribution histograms of sorted normal human leukocytes stained with acridine orange: (A) erythrocytes and leukocytes, and (B) sorted leukocyte fractions 1–4. Sorted fractions 1–4 are granulocytes, monocytes, large lymphocytes, and small lymphocytes, respectively.

Figure 5 shows three examples of green/red fluorescence ratio distributions obtained from different normal human subjects, with distinct differences between the two lymphocyte percentages. For example, peak 3 of Fig. 5A which consists of the larger class of lymphocytes is almost nonexistent. Al-

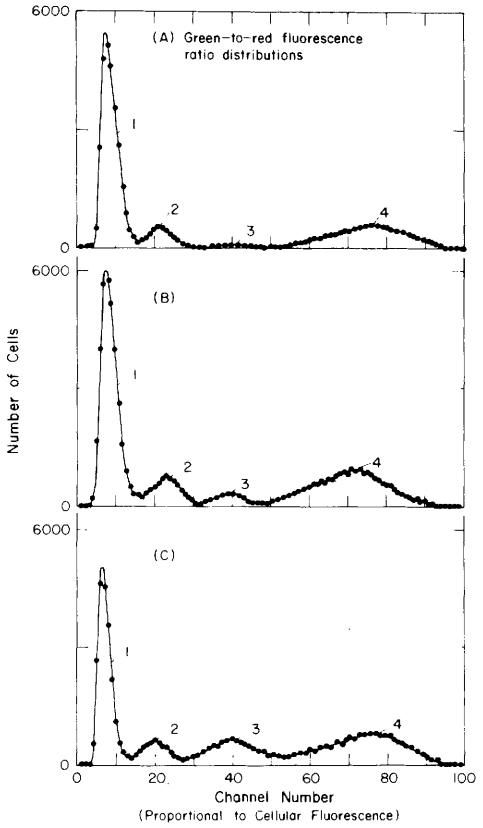

FIG. 5. Green-to-red fluorescence ratio frequency distribution histograms of acridine orange-stained leukocytes from three normal humans.

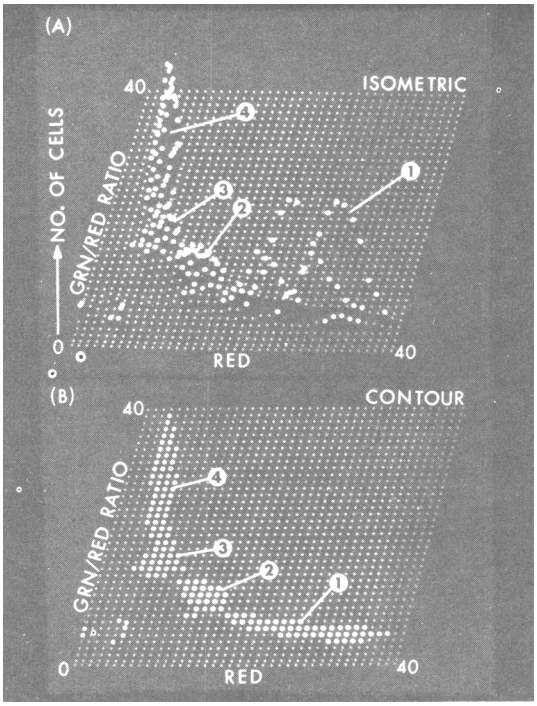

FIG. 6. Two parameter red fluorescence and green/red fluorescence ratio plots of acridine orange-stained normal human leukocytes: (A) three-dimensional frequency distributon histogram, isometric plot, and (B) two-dimensional contour view. Regions 1–4 consist of granulocytes, monocytes, large lymphocytes, and small lymphocytes, respectively.

ternatively, peak 3 of Fig. 5C is somewhat elevated and consists of approximately 18% of the total leukocyte population. About 35% of the leukocytes are contained under peak 4. Figure 5B is characteristic of typical green/red fluorescence ratio distributions with approximately 7 and 33% of the total leukocyte population contained within peaks 3 and 4, respectively. Computer curve-fitting techniques could be used to accurately determine the relative numbers of lymphocytes contained within peaks 3 and 4 on an automated basis for studying lymphocyte kinetics under stress conditions.

Figure 6 shows a typical three-dimensional frequency distribution histogram (isometric display) and two-dimensional contour view of two parameter red fluorescence and green/red fluorescence ratio relationships in human leukocytes. The isometric display provides a method to quantitate the number of leukocytes within a given range of two cellular properties. Examination of this display shows four separate regions of peaks, consisting of granulocytes, monocytes, and the two types of lymphocytes. The contour view, which is a base-plane sectional view of the isometric display, better illustrates the relationship between red fluorescence and the green/red fluorescence ratio with the four leukocyte regions vividly shown. The two lymphocyte types (regions 3 and 4) have nearly overlapping red fluorescence values but well separated green/red fluorescence ratios as viewed with respect to each axis. These results correspond well with the individual red fluorescence and green/red fluorescence ratio distributions of Fig. 2. If coupled with cell sorting, this method of analysis provides additional capability to characterize

leukocytes on the basis of two or more parameters.

Summary. We have demonstrated that normal human peripheral leukocytes vitally stained with acridine orange can be separated into four groups, consisting of granulocytes, monocytes, and two types of lymphocytes, on the basis of green/red fluorescence ratio measurements which characterize nuclear and cytoplasmic staining properties. Evaluation of the two sorted lymphocyte populations has suggested two morphologically and functionally distinct subclasses which are normally classified as large and small lymphocytes. When considered by volume measurement alone, small and large lymphocytes were not discrete classes but had overlapping distributions. However, the fluorescence ratio distributions clearly indicated that these are discrete classes of cells rather than representing an arbitrary classification of a continuum.

Since green nuclear fluorescence is nearly uniform, the green/red fluorescence ratio measurement permits characterization of leukocytes as an inverse cytoplasmic granulation density function. Using these criteria, granulocytes characterized by coarse cytoplasmic granulation had lower green/red fluorescence ratio signal amplitudes than lymphocytes which have finer granulation. The red/green fluorescence ratio distributions also have been measured, but the degree of separation between the two lymphocyte types was less pronounced.

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