Discrimination of Human Leukemia Subtypes by Flow Cytometric Analysis of Cellular DNA and RNA

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A newly developed flow cytometry technique for simultaneous measurements of three features of individual cells—DNA, RNA, and nuclear diameter—using acridine orange as a fluorescent metachromatic dye, has been applied to cell-cycle analysis, DNA stemline determination, and to classification of 102 cases of human leukemias in adults. Acute lymphoblastic leukemia (L1–2) was characterized by moderately increased RNA of G_{0}G_{1} cells as compared to normal lymphocytes; acute nonlymphoblastic leukemia (M 1–5) by very high RNA of G_{0}/G_{1} cells. Both had either diploid or aneuploid DNA stemlines. Chronic lymphocytic leukemia showed diploid DNA, very low proliferation, and low RNA, similar to that found by us to be typical for normal B cells. In chronic myelogenous leukemia, two cell populations were distinguished, one with high RNA, the other with very low RNA and elongated nuclear diameter due to stripped, unfolded nuclei of polymorphonuclear leukocytes. The number of leukemic blast cells, identified by aneuploid DNA values, correlates well with conventional microscopy counts and could be followed during the course of treatment. Thus, acridine orange flow cytometry can be used to discriminate subtypes of human leukemias, to determine cell cycle stages, and to detect and monitor aneuploid leukemia stemlines.

Recent advances in cytostatic therapy of the acute leukemias emphasize the critical role of a precise diagnosis and cell classification in the choice of treatment regimens. For instance, in acute lymphoblastic leukemia (ALL), high remission rates, long remission duration, and possible cures can be achieved, especially in children.\(^1^4\) Acute myeloblastic leukemia (AML) is more difficult to treat, as is the blastic phase of chronic myelogenous leukemia (CML), which responds poorly to chemotherapy although cases with lymphoid transformation have been described that respond temporarily.\(^5^7\) Morphology alone is not sufficient for precise classification of the leukemias.\(^8^9\)

The molecular basis for drug actions that selectively favor one cell type over another is presumably related to differences in biochemical constituents as well as proliferative and functional states of the cell. Thus, a better understanding of the differences between normal and leukemic cell types and the nature of their interaction with drugs would help in planning new chemotherapeutic protocols.

With present techniques of flow cytometry, it is possible to investigate some of the biochemical properties of intact, individual cells at very rapid rates.\(^10^12\) to determine features that distinguish subclasses of cells,\(^13^14\) and to evaluate their response to drugs.\(^15^16\)

In this study we measured, simultaneously, the content of stainable ribonucleic (RNA) and deoxyribonucleic acid (DNA) of individual mononuclear cells\(^7\) from normal and leukemic peripheral blood and bone marrow. These measurements provide: (1) an estimate of ploidy levels; (2) frequency distributions of normal and leukemic cells in the different phases of the cell cycle (G_{0}/G_{1}, S, G_{2} + M); (3) mean values of stainable RNA and DNA for various subpopulations; (4) an estimate of RNA/DNA ratios with regard to the different cell-cycle phases; and (5) discrimination of subpopulations according to ploidy (DNA) and RNA accumulation, which might be related to transcriptional activity. These data are useful for the diagnosis and classification of certain leukemias.\(^18\) They are also helpful for the subclassification of certain morphologically uniform leukemic cells and normal lymphocytes\(^19\) and provide a means to determine the relative number of leukemic cells, potentially to detect relapse at an early stage.

At this time we report on 102 cases of leukemia studied by this technique.

MATERIALS AND METHODS

Preparation of Cells

Heparinized bone marrow and peripheral blood specimens were obtained from normal volunteers and from leukemic patients prior to and at intervals during therapy. Blood or bone marrow was mixed with equal volumes of phosphate-buffered saline (PBS) containing 2 mM MgCl\(_2\) and layered on 5 ml Lymphoprep (density 1.077 g/ml) (Accurate Chemical and Science Corp., Hicksville, N.Y.). The suspension was then centrifuged 20 min at 1000 g at room temperature. Erythrocytes and mature myeloid cells are in the pellet, while lymphocytes, monocytes, bone marrow spicules, and dividing myeloid cells are found in the interphase above the Lymphoprep layer.\(^16\) The interphase was aspirated with a Pasteur pipet, filtered through 33 μ nylon mesh filters to remove marrow spicules and clumped cells, washed twice in PBS plus 2 mM MgCl\(_2\), and then suspended in a medium containing PHS, 2 mM MgCl\(_2\), and 10% fetal calf serum.

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In order to obtain pure monocytes or to remove monocytes from a lymphocyte-monocyte mixture, two different methods were used.

1. Lymphoprep-separated lymphocytes and monocytes were washed in PBS and then incubated for 1 hr with tissue culture medium (Basal medium (Eagle) with 25 mM Hepes buffer (GIBCO, Grand Island, N.Y.)), supplemented with 20% fetal calf serum, on Falcon Petri dishes. Monocytes adhere to the plastic surface and can be removed with Trypsin-EDTA (Flow Laboratories, Rockville, Md.) and measured for DNA-RNA content.

2. Lymphoprep-separated lymphocytes and monocytes were incubated for 1 hr at 37°C with carbonyl-iron. Cells that phagocytized carbonyl-iron were separated by another Lymphoprep separation procedure. The interphase then consists of pure lymphocytes that can be measured in the flow cytometer.

Granulocytes were obtained from healthy donors after enrichment in a Hemoctics cell separator (kindly provided by Dr. L. Reich) and suspended in hydroxyethyl starch. They were further separated from lymphocytes by the standard Lymphoprep procedure.

**Simultaneous Staining for DNA and RNA**

Acridine orange (AO) was used for simultaneous staining of RNA and DNA in unfixed cells as described in detail by Traganos et al. With this technique, AO intercalates into double helical nucleic acids (DNA) and fluoresces green (530 nm) upon excitation with blue light, while it stacks on single-stranded nucleic acids (RNA) to fluoresce red (640 nm). Since a large amount of RNA in situ is double-stranded, it may be selectively denatured to the single-stranded form prior to staining. This is accomplished by moderate heat or with chelating agents like EDTA. The unfixed cells are made permeable to AO and chelating agents by short pretreatment with detergents, like Triton X, at low pH.

Specifically, the staining procedure was as follows. Aliquots of 0.2 ml of cells suspended in PBS, 2 mM MgCl₂, and 10% fetal calf serum were mixed with 0.4 ml of a solution containing 0.1% (v/v) Triton X-100 (Sigma Chemical Co., St. Louis, Mo.), 0.08N HCl, and 0.15M NaCl. After 30 sec, 1.2 ml of AO (Polysciences Inc., Warrington, Pa., chromatographically purified) (6 µg/ml) in 10⁻³M EDTA, 0.15M NaCl, 0.1 M phosphate-citrate buffer, pH 6.0, was added, resulting in a final dye concentration of 1.3 · 10⁻³M AO. Measurements were performed within the next 10 min. In order to check the specificity of RNA staining, RNase A (Worthington Biochemical Corp., Freehold, N.J.) was added (10⁻⁵U/ml) to the stained cell suspension at room temperature. Measurements were performed prior to and 15 min after RNase treatment at exactly the same machine settings.

**Instrumentation and Data Processing**

The flow cytometer used was a cytofluorograf FC 200 (Ortho Diagnostic Instruments, Westwood, Mass.) with flat-windowed flow channel of square cross-section and an elliptically focused argon-ion laser emitting at 488 nm. Two wavelength bands of fluorescence, green at 515–575 nm (denoted F₅₃₀) and red at 600–650 nm (denoted F₄₆₀), were selected by appropriate filters, measured simultaneously by separate photomultipliers, subtracted from background fluorescence, and the integrated values digitized in a 10-bit analog/digital converter and recorded by an interfaced Nova 1220 minicomputer (Data General, Southboro, Mass.). A third parameter, F₅₃₀ pulswidth, i.e., the time taken by each cell nucleus (fluorescing green) to pass through the focus of the laser beam, was also recorded and used to distinguish single cells from cell aggregates as well as to estimate nuclear size. The term “GPW” will be used for F₅₃₀ pulswidth; “DNA” for F₄₆₀; “RNA” for F₅₃₀.

A total of 5000 cells were examined per specimen at rates of 200/sec, and the 3 measurements for each cell recorded on magnetic disk. Data analysis was performed with the help of a Tektronix 4010-1 graphic terminal using computer programs developed by Sharpless in our laboratory. All figures shown are produced by these computer programs from the raw data and obtained from a Tektronix hard copy unit linked to the graphic terminal.

It should be emphasized that some experiments were deliberately performed at different machine settings. Thus, while hydrodynamic and optical adjustments always were made to obtain maximal resolution, as determined by minimizing the coefficient of variation, the sensitivity of F₆₆₀ and F₄₆₀ photomultipliers (PMT) were varied deliberately over a large range. Typical measurements on normal mononuclear blood cells were performed at a red PMT gain approximately ten times as high as the green PMT gain; while typical measurements on leukemic cells were performed at a red PMT gain approximately six times as high as the green PMT gain. For convenience, these settings were varied from experiment to experiment according to the differences in RNA content between normal and some leukemic cells. However, when mononuclear cells of normal blood were compared to leukemic blast cells in order to obtain the RNA-Index (RI), all measurements were done at the same settings.

**RNA-Index (RI)**

In order to normalize the data and calibrate the RNA distribution of populations of abnormal blood and bone marrow cells, the mean value of RNA (F₆₆₀) for quiescent mononuclear peripheral blood cells (i.e., lymphocytes with G₀₁ DNA content) was used as a reference. Relative RNA values (RNA-Index: RI) were then expressed as the ratio of the mean RNA value of the G₀₁ cells of the sample, times 10, divided by the median RNA value of the reference mononuclear blood cells with G₀₁ DNA content. Measurements were carried out under the same staining conditions and optical and electronic adjustments.

\[
\text{RNA-Index (RI)} = \frac{\text{Mean } G₀₁ \text{ RNA of sample}}{\text{Median } G₀₁ \text{ RNA of control}} \times 10
\]

Thus, normal peripheral blood lymphocytes have an RNA index of 10; separated B lymphocytes, which have less RNA than T lymphocytes, have an RNA-Index in the range of 8 (see Results, below). Cells with G₀₁ DNA were chosen for RNA evaluation and comparison because of the increase in RNA per cell during progression through the cell cycle.

**RESULTS**

**Normal Peripheral Blood**

The distribution of F₅₃₀ values of mononuclear cells separated from peripheral blood of normal healthy donors is unimodal, indicating that all cells have normal diploid DNA content, denoted 2.0 c. The distribution is Gaussian with a coefficient of variation (CV) of 2.6%–3.0% in our system. The number of cells in S and G₂ + M is below 1%. The F₆₆₀ distribution is heterogeneous, with at least two different populations, demonstrating considerable sample-to-sample variation in size of each component. Removal of monocytes with carbonyl-iron results in two pronounced peaks (Fig. 1, top), suggesting that monocytes have an intermediate level of red fluorescence. After treatment with RNase there is a shift to lower red fluorescence.
Lymphocytes

Monocytes

Fig. 1. Computer-drawn histograms representing red fluorescence values ($F_{>600}$) of AO-stained human peripheral blood leukocytes (abscissa = $F_{>600}$, ordinate = relative frequency). The upper histogram represents lymphocytes, the lower histogram represents monocytes separated by adherence to plastic surface.

Monocytes isolated by adherence to a plastic surface demonstrate a unimodal distribution that is skewed towards high red fluorescence values with an intermediate mean red fluorescence (Fig. 1, bottom). Thus, the peaks seen in $F_{>600}$ histograms are related to subclasses of lymphocytes and to monocytes. Enriched populations of T and non-T cells, as well as IgM and IgG receptor-bearing T cells (T$_M$, T$_Y$) were previously shown to differ in mean content and distribution of $F_{>600}$. B lymphocytes have a low mean $F_{>600}$ value with skewed distribution toward higher values. The majority of T lymphocytes, namely T$_M$ or "helper" cells, have about twice the $F_{>600}$ value of B and T$_Y$ ("suppressor") cells.

Granulocytes can be distinguished from lymphocytes and monocytes by differences in their red and green fluorescence as well as by markedly higher green pulselwidth (GPW) values. Figure 2B shows the red and green fluorescence of a granulocyte–lymphocyte mixture obtained from the Hemonetics machine. Two populations are distinguishable. Cells with low red and high green fluorescence (Fig. 2B, arrow) are identified as granulocytes, since they are lost after removing granulocytes with the Lymphoprep technique (Fig. 2 C and D). Microscopic examination of the samples after Lymphoprep separation confirms that almost all granulocytes are removed.

The measurements by flow cytometry can be explained as follows. The pretreatment with detergent in the acridine orange staining technique used here and the shearing forces in the flow cytometer disrupt the granulocyte cell membranes, strip and extend the multisegmented nuclei, and align them in the direction of flow in the instrument. Lymphocytes and monocytes remain intact. The lengths of the unfolded nuclei are measured as green fluorescence pulselwidths (GPW), with increased length giving rise to higher GPW values. The slightly higher green fluorescence of granulocyte nuclei, compared with lymphocytes and monocytes, may be the result of a greater yield in the fluorescence measurements of the unfolded nuclei. The low red fluorescence, of course, results from the loss of cytoplasm. These changes in granulocytes are dependent on the serum concentration and can be minimized by addition of 30% serum or cell fixation (30).

Fig. 2. Identification of granulocytes by AO flow cytometry. Computer-drawn histograms displaying green ($F_{>600}$) fluorescence, red ($F_{red}$) fluorescence, and green pulselwidth (GPW). White blood cells prior to (A and B) and after (C and D) Lymphoprep separation. (A) Granulocytes (arrow) have increased $F_{>600}$ and GPW values; they are removed by the separation procedure (C). (B) Granulocytes have increased $F_{>600}$ and low $F_{red}$ values; compare with (D) after they are removed by the separation procedure.

Normal Bone Marrow

Bone marrow from normal donors demonstrates the pattern shown in Fig. 3. The joint distribution of $F_{>30}$...
Normal Bone Marrow

Fig. 3. Computer-drawn F_{530} ("DNA")-F_{630} ("RNA") histograms of normal bone marrow cells after Lymphoprep separation and acridine orange staining. The presence of G_{0/1} cells with different amounts of stainable RNA indicates that some cells have increased RNA values, but G_{0/1} DNA content. Cells with higher DNA content are in S and G_{2} + M.

ALL

Fig. 4. Computer-drawn DNA-RNA histogram of acute lymphoblastic leukemia (ALL) cells prior to treatment. (A) Peripheral blood. The small peak represents cells with diploid DNA content; the higher peak consists of hyperdiploid cells in G_{0/1}. Few cells are in S and G_{2} + M (arrow = diploid peak). (B) Bone marrow. Diploid and hyperdiploid peaks as in (A). More cells are in S (10.5%) and G_{2} + M. The G_{0/1} peak of the proliferating leukemic population has a higher DNA and RNA content as compared to the remaining normal diploid cells. RNA increases during progression through the cell cycle. The two minor populations at the edge of the histogram represent debris and isolated nuclei. (C) Hyperdiploidy of the leukemic stemline is proved by mixing leukemic blood and normal lymphocytes. Increase of the previously lower peak is due to an increase of normal diploid cells.
of G₁ lymphoblasts as compared to normal lymphocytes. As an additional feature, this patient shows a hyperdiploid DNA stemline of his leukemia.

The F₃₅₀−F₆₀₀ histogram of peripheral blood cells (Fig. 4A) shows two peaks that differ in both F₃₅₀ and F₆₀₀ values. The smaller peak is due to normal, diploid lymphocytes (2c) (arrow), as proven by the increase in that peak following the addition of normal lymphocytes to the leukemic peripheral blood cells (Fig. 4C, arrow). The larger peak is due to lymphoblasts, which are the predominant cell type. The F₅₃₀ value of the lymphoblasts is 19% higher (2.38c) than normal (2.0c); the F₆₀₀ value is 36% higher. The RNA-Index (RI) is 13.8. The histogram of ALL bone marrow (Fig. 4B) shows a pattern similar to peripheral blood, though with more cells having higher F₃₅₀ and F₆₀₀ values, namely cells in S and G₂ + M; 10.5% of the hyperdiploid cells are computed to be in S. The pulse ³H-TdR-labeling index of the lymphoblasts is 10.0% (kindly provided by Dr. Z. Arlin).

Statistical analysis of the 26 cases of ALL investigated is given in Table 1. The mean RNA-Index is 11.8 ± 2.0; the number of cells in G₀/₁ = 91.9%, S = 5.1%, and G₂ + M = 3.0%. Aneuploidy was found in 4 cases (15.4%), and repetitive measurements were performed during treatment and at time of relapse. The correlation between determination of blasts by microscopy on smears and flow cytometric determination of aneuploid cells is excellent (r = 0.953) and shown in Fig. 5.

Acute Nonlymphoblastic Leukemias

The following typical example is chosen from 46 adult patients with acute nonlymphoblastic leukemia (ANLL) who have been studied to date. They were classified as M 1–5, according to the FAB classification.⁹

The F₃₅₀−F₆₀₀ histogram of the bone marrow (BM) of a patient with acute promyelocytic leukemia (diagnosis established by morphology and cytochemistry) shows two peaks differing slightly in F₃₅₀ and considerably in F₆₀₀ values (Fig. 6, top). The first peak (11.5% of all cells) consists of diploid (2.0c) bone marrow cells (RI = 8.6) that correspond to the 10.5% lymphocytes counted in bone marrow smears. Normal lymphocytes (Co), measured under identical conditions, are shown for comparison in the lower panel of Fig. 6. The majority of bone marrow cells (88.5%) have markedly increased F₆₀₀ values (RI = 21.2), are slightly hyperdiploid (2.1c), and correspond in relative number to the promyelocytes counted in the smear. Cell-cycle analysis shows good correlation of cells in S phase by flow cytometry (3.5%) with ³H-TdR-labeling index (LI = 2.2%). The GPW of the cells with high F₆₀₀ values is 37 AU as compared to 35 AU of control lymphocytes, indicating bigger nuclei for the blast cells. Treating bone marrow cells with RNase led to a decrease of the mean F₆₀₀ value from 51.7 to 24.2 AU (53.2%).

Statistical analysis of the 46 cases of ANLL investigated is given in Table 1. The mean RNA-Index is 20.6 ± 4.0, calculated for the leukemic blast cells; the number of cells in G₀/₁ = 93.5%, S = 4.1% and G₂ + M = 2.4%.

There was good correlation between the percentage of blast cells identified by high RNA content by flow

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### Table 1. Cell-Cycle Analysis and RNA-Indices of Leukemias and Normal Bone Marrow

<table>
<thead>
<tr>
<th></th>
<th>ALL (n = 26)</th>
<th>ANLL (n = 46)</th>
<th>CLL (n = 15)</th>
<th>CML (n = 15)</th>
<th>Normal BM (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G₀/₁</td>
<td>91.9 ± 6.3</td>
<td>93.5 ± 3.6</td>
<td>99.5 ± 2.9</td>
<td>92.4 ± 3.3</td>
<td>90.0 ± 4.5</td>
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<tr>
<td>S</td>
<td>5.1 ± 4.1</td>
<td>4.1 ± 2.3</td>
<td>0.3 ± 0.2</td>
<td>4.8 ± 2.5</td>
<td>6.7 ± 2.6</td>
</tr>
<tr>
<td>G₂ + M</td>
<td>3.0 ± 2.8</td>
<td>2.4 ± 1.7</td>
<td>0.2 ± 0.2</td>
<td>2.7 ± 1.5</td>
<td>3.3 ± 2.1</td>
</tr>
<tr>
<td>CV</td>
<td>4.8 ± 1.9</td>
<td>5.2 ± 1.2</td>
<td>3.7 ± 0.7</td>
<td>5.3 ± 0.4</td>
<td>5.2 ± 0.3</td>
</tr>
<tr>
<td>RI</td>
<td>11.8 ± 2.0</td>
<td>20.6 ± 4.0</td>
<td>8.6 ± 0.7</td>
<td>11.9 ± 2.6*</td>
<td>11.3 ± 0.6*</td>
</tr>
</tbody>
</table>

n = Number of patients; G₀/₁, S, G₂ + M = percentage of cells in the different cell cycle phases ± SD; CV = coefficient of variation of G₀/₁ DNA measurements; RI = RNA-Index (see Materials and Methods).

*Mean of a bimodal distribution.
cytometry and by light microscopic examination of smears, as shown in Fig. 7 (r = 0.751). So far, no consistent differences have been found between myeloblastic, promyelocytic, myelomonocytic, and monocytic acute leukemias. Aneuploidy was found in 8 patients (17.4%) and studied serially during treatment.

The correlation between aneuploid ANLL cells as determined by flow cytometry and blast cells counted on bone marrow smears is shown in Fig. 5.

**Chronic Lymphocytic Leukemia**

The following typical example represents 15 cases of chronic lymphocytic leukemia (CLL) examined by us.

The $F_{330} - F_{400}$ histogram of peripheral blood shows 99.5% of the cells in $G_{0/1}$, 0% in S, and 0.5% in $G_2 + M$ (Fig. 8). $F_{330}$ content was diploid. The $F_{400}$ distribution was very similar to that of normal B cells with a low mean value, slightly skewed to the right. The RNA-Index was 8.3. The histogram of bone marrow cells was virtually indistinguishable from that of peripheral blood. A comparison between normal and abnormal lymphocytes reveals virtually no differences between normal B cells and CLL lymphocytes. The RNA index is 8.0 for the normal non-T cells and 8.6 ± 0.7 for the CLL cells. Cell-cycle distribution and RNA-Index of 15 cases is listed in Table 1.

**Chronic Myelogenous Leukemia**

The following typical example is chosen from 15 cases of chronic myelogenous leukemia (CML) in adults.

The $F_{330}$, $F_{400}$, and GPW histograms of CML
marrow and control lymphocytes (CO) measured under identical conditions are shown in Fig. 9. The leukemic cells are Ph¹ positive and diploid (courtesy of Dr. R. S. K. Chaganti), and at this time, bone marrow (BM) contained 81.5% myeloid cells. The F₃₅₀ histogram (Fig. 9, DNA) shows an apparent aneuploid G₁ peak (2.3c), but this is due to increased F₃₅₀ of the unfolded, elongated granulocyte nuclei (compare with Fig. 2). The granulocyte nuclei can be recognized and separated from intact myeloid cells by the RNA histogram (Fig. 9, RNA), which shows two peaks, one at very low F₄₆₀₀ (R1 = 6.1; unfolded granulocyte nuclei) and the other at very high F₄₆₀₀ (R1 = 19.6; myeloid cells). The third feature, nuclear diameter (GPW), shows a distribution skewed towards higher values, also because of the unfolded granulocyte nuclei.⁰ The histograms of peripheral blood cells from this patient were similar to those of the bone marrow. Despite removal of a majority of granulocytes by the standard separation technique in this and the other CML cases studied, there were still enough polymorphonuclear leukocytes remaining to result in the bimodal distribution described above and characterizing this type of leukemia. Statistical analysis of 15 cases of CML is given in Table 1.

In summary, the flow cytometry findings in CML are characterized by the presence of two populations of G₁ cells, one with high F₄₆₀₀ value (myeloid cells) and the other with low F₄₆₀₀ and abnormally high GPW (stripped, unfolded nuclei of granulocytes).

RNA-Index and Cell-Cycle Data in Differential Diagnosis

The RNA-Indices of the different leukemias and normal bone marrow are plotted in Fig. 10; a complete statistical analysis of the DNA and RNA measurements is given in Table 1.

No differences were found among G₀/₁, S, and G₂ + M frequencies in ALL and ANLL, but there was a nonoverlapping difference in RNA-Index: 11.8 ± 2.0 for ALL and 20.6 ± 4.0 for ANLL. CLL has an extremely low number of S and G₂ + M cells and the lowest RNA-Index. The RNA discrimination of CML and normal bone marrow is bimodal; the numbers given represent the mean RNA-Indices of all G₀/₁ cells.

DISCUSSION

Flow cytometry was first applied to the study of leukemias by Buechner et al.⁴³,⁴⁴ using ethidium bromide for DNA measurements, and by ourselves⁴⁵,⁴⁶ using a supravital acridine orange stain that was developed earlier by Adams and Kamentsky⁴⁷,⁴⁸ for automated differential leukocyte counts.⁴⁹,⁵⁰ The supravital acridine orange technique simultaneously...
FIG. 9. Computer-drawn DNA, RNA, and GPW (green pulsewidth) histograms from chronic myelogenous leukemia (CML) bone marrow cells (top) and control lymphocytes (bottom). The DNA histogram shows a change in the position of the G01 peak (for explanation see text) and low proliferation. The RNA histogram is bimodal, with lower and higher values than those found in normal lymphocytes. The GPW histogram shows a unimodal distribution, slightly greater than normal.

measured AO binding to DNA and dye uptake by lysosomes. A number of DNA-specific stains were subsequently applied to the study of leukemias by others.

Recently, we have devised acridine orange staining techniques for RNA in fixed cells and for DNA and RNA simultaneously in detergent-treated unfixed cells. This latter staining procedure made it possible to measure three parameters per cell simultaneously—DNA, RNA, and nuclear diameter—now permitting subclassification of the leukemias by flow cytometry. Unlike the supravital staining technique used previously with AO, dye uptake does not rely on lysosomal function. The unfixed cells are pretreated with Triton X-100 at low pH to make their cytoplasmic membrane permeable to the dye; at that pH, nucleic acids remain insoluble. Subsequent staining with AO in the presence of a chelating agent (EDTA) results in the denaturation of any double-stranded RNA. The dye intercalates within double helical DNA where it is maintained in monomeric form that fluoresces orthochromatically green (F530); single-stranded RNA stains metachromatically red (F640) due to “stacking” of dye molecules bound electrostatically to phosphate groups. The method was recently reviewed by Darzynkiewicz.

DNA

The DNA content provides information on cell ploidy and on the position of each cell in the cell cycle. Estimates of the percent synthesizing cells by flow cytometry correlate well with those obtained by 3H-thymidine pulse labeling, autoradiographic labeled mitotic curves, and 14C-3H-thymidine double-layer autoradiography. The specificity of the AO staining technique for DNA used by us can be shown by DNase, which removes more than 90% green fluorescence (F530) while not significantly affecting red fluorescence (F640). Further, stoichiometry of this DNA stain was confirmed recently by Coulson et al. over the entire range of 3.5–91 pg DNA/cell. In our experiment, G0 + M peaks are twice the G1 peaks, and

RNA-INDEX IN LEUKEMIA

![RNA-INDEX IN LEUKEMIA](image)

FIG. 10. RNA-indices derived from DNA-RNA histograms of patients with acute lymphoblastic (ALL), acute nonlymphoblastic (ANLL), chronic lymphocytic (CLL), and chronic myelogenous (CML) leukemias. The bimodal RNA distributions typical for CML and normal bone marrow were expressed as mean RNA-indices for all G01 cells. Of interest, the one patient with CML and very high RNA index entered the blastic phase of CML shortly thereafter.
aneuploid stemlines identified by AO have been confirmed by karyotyping or by flow cytometry using ethidium bromide and DAPI (data not shown).

In some cases, karyotypes cannot be determined because proliferative rates are too low to yield an adequate number of mitoses. Since flow cytometry is independent of proliferative activity, it may be possible with this technique to identify aneuploid stemlines that would otherwise be overlooked. Barlogie et al. reported DNA deviations in some cases with normal karyotypes, and we had similar results in childhood ANLL. This apparent discrepancy was believed to be due to a lack of proliferation of the aneuploid leukemic clones. The clinical significance of the finding, of course, lies in the possibility of a different response to a given drug regimen by different clones. For example, Rosenthal et al. recently reported differences in response to chemotherapy of hypodiploid and hyperdiploid CML in blastic crisis.

In leukemias with aneuploid DNA stemlines, it is possible to identify the leukemic blasts by their DNA content. In this series of adult acute leukemias, 16.7% were aneuploid, which equals the 16.6% aneuploidies reported by Barlogie et al. In childhood ANLL, we found 3/5 aneuploid cases. The correlation between flow cytometry counts obtained in this way and counts by light microscopy is excellent (Figs. 4 and 5) (r = 0.953). Furthermore, it is possible to follow these patients during treatment, and with flow cytometry counts of 10^4 and 10^5 cells per sample, to identify small numbers of leukemic cells with much better statistical reliability than by light microscopy. The equation given in Fig. 5 indicates that 9% aneuploid cells are detected even if no leukemic blast cells are seen in smears. This could influence the design of chemotherapeutic maintenance schedules and permit early detection of relapse.

The S-phase fractions (5.1% for ALL, and 4.1% for ANLL) are similar to those found in 94 cases of childhood acute leukemia by Murphy et al. and Vogler et al., but lower than in other series. This seems to be due primarily to admixture of peripheral blood blast with low proliferation. Hiddeman et al. found 60% higher S-phase fraction and labeling index in marrow biopsies as compared to marrow aspirations.

There have been conflicting reports of the prognostic significance of S-phase cells, as determined by flow cytometry or autoradiography, and further investigation of this parameter still is necessary. It is possible, for example, that a change in 3H-thymidine labeling index or S-phase cells by flow cytometry after initiating chemotherapy will be more important than the pretreatment levels. The effects of drugs on the cell cycle also can be determined rapidly and easily with flow cytometry, so it is potentially useful as a clinical tool for monitoring treatment effect.

**RNA**

The part of the red fluorescence (F_r) after incubation with RNase is presumed to be due to binding by RNA and accounts for 35% of F_r for leukocytes (Fig. 1) and 53% for ANLL cells. The remaining F_r is attributed to nonspecific binding by other macromolecules. Bauer and Dethlefsen recently compared red fluorescence of AO-stained cells with biochemical determination of RNA in different cell systems and found a correlation coefficient of 0.994 (personal communication). Biochemical evidence suggests that about 80% of the total RNA normally is ribosomal. Butorelli and coworkers report an accumulation of large amounts of double-stranded heterogeneous RNA in the nuclei of leukemic cells and have suggested that this defect in RNA processing is a basic mechanism in leukemogenesis.

We reported earlier that B and T (μ,γ) lymphocytes differ in mean value and distribution of F_r. B cells of CLL are almost identical to normal non-T (predominantly B) cells and, like them, have a low RNA value (RI = 8.6) and extremely low proliferative rates by DNA histograms (Table 1, Fig. 8). Thus, it is possible to distinguish B-cell CLL from the other leukemias we have studied and to monitor the RNA pattern of leukocytes during therapy until a "normal" pattern returns.

The G1 cells of acute lymphoblastic leukemia have an increased RNA content compared to normal lymphocytes. This is reflected by an RI of 11.8 (Table 1). However, considerably higher RNA values (RI = 20.6 ± 4.0) are found in the G1 cells of ANLL, and in all cases of ANLL with an adequate sampling, the RI was higher than in any case of ALL (see Fig. 10). In ANLL, the number of leukemic blasts on smears correlated well with the percentage of cells with high RNA content (Fig. 7, r = 0.751). The equation given in Fig. 7 indicates that 21% cells with high RNA content are measured even in the absence of leukemic blasts. This is due to normal hemopoietic cells, especially myeloid cells, with high RNA content.

The differentiation between CLL, ALL, and ANLL by this flow cytometry technique is quite striking and consistent. Results can be obtained within 1–2 hr, offering a considerable clinical advantage over the more time-consuming determinations of terminal deoxynucleotidyl transferase and granulocyte colony-forming units. It is well to emphasize that the RNA index determined by flow cytometry refers only to cells in G1 phase of the cycle. This differs from a
biochemical assay that would reflect increases in RNA related to the proliferative state of the cells, and even with a pure population of leukemic cells, could not be expected to differentiate between ALL and ANLL.

Nuclear Pulsewidth (GPW)

A third feature measured by us, simultaneous with the fluorescence measurements of DNA and RNA, is the duration of the F₅₃₀ fluorescence pulse as each cell passes through the narrowly focused laser beam. This provides a measure of the nuclear diameter of the cell, which increases slightly with its progress through the cell cycle. It also permits rejection of cell doublets and higher aggregates. In addition, the nuclear pulsewidth measurements facilitate the identification of any mature granulocytes that may be present. The presence of two cell populations, one with low red fluorescence and high GPW, the other with very high red fluorescence, characterizes CML. The technique, therefore, is useful for monitoring of CML and the detection and rapid discrimination of lymphoblastic and myeloblastic leukemic cell types in the terminal blastic phase of the disease.

A continuation of the current work will provide a larger sample size and permit us to correlate these data with other studies, e.g., granulocyte colony-forming units (CFUc), immunologic surface markers, terminal deoxynucleotidyl transferase, labeling index, and karyotype analysis. An improved classification and understanding of the acute and chronic leukemias with regard to proliferation and differentiation will be possible, and effects of therapy can be monitored with this new technique, including counts of leukemic cells well below that possible by ordinary light microscopy.

ACKNOWLEDGMENT

The authors wish to thank the attending staff of the Leukemia/Lymphoma Service, Memorial Hospital, for their cooperation in supplying samples and clinical information; S. McKenzie for the cytochemical studies; J. Steinmetz for excellent technical assistance; and R. Nager for her assistance in the preparation of this manuscript.

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