Flow Analysis of DNA Content and Cell Size in Non-Hodgkin's Lymphoma

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ABSTRACT

Cellular DNA content, Coulter volume, and light scatter were measured in cell suspensions from 30 non-Hodgkin's lymphomas in order to assess flow analysis as a quantitative and reproducible means of evaluating these diseases. Nonneoplastic control populations included 31 samples obtained from lymph nodes, spleens, tonsils, and peripheral blood. Cellular DNA and light scatter were measured on ethanol-fixed cells by flow microfluorometry using nuclei isolated from chicken erythrocytes as an internal standard. For DNA analysis, the cells were stained with propidium iodide following RNase treatment. The cellular DNA content of the human populations was expressed as a ratio between the DNA content of the human G0—G1 cells and that of the chicken erythrocyte nuclei. The mean DNA ratio for the 31 nonneoplastic samples was 2.83 ± 0.08 (S.D.). In these samples, the coefficient of variation of the human G0—G1 peak ranged from 2.27 to 3.63% (mean 3.09 ± 0.32%). Fifteen of 30 non-Hodgkin's lymphomas, including 7 of 15 low-grade lymphomas and 8 of 15 high-grade lymphomas, had abnormal DNA content, the coefficient of variation of the human G0—G1 peak, corrected for differences in instrument setting was greater than that seen in the nonneoplastic populations. A good correlation between the percentage of cells calculated to be in the S phase of the cell cycle and the expected clinical behavior of the tumors was observed. In those lymphomas in which the S-phase percentages could be calculated, 11 of 13 low-grade lymphomas had less than 5% of the cells in S phase, and 7 of 10 high-grade lymphomas had greater than 5% of the cells in S phase. Thirteen of 21 neoplastic cases in which Coulter volume determinations were performed could be distinguished from the nonneoplastic controls on the basis of their modal volume. Although some correlation was observed between light scatter of ethanol-fixed cells and Coulter volume measurements on unfixed cells, light scatter was found to be less discriminatory. Altogether, by all three flow parameters studied, 26 of 30 (87%) of the neoplastic cases could be distinguished from nonneoplastic controls.

INTRODUCTION

The non-Hodgkin's lymphomas are a heterogeneous group of neoplasms with different presentations, clinical courses, and responses to therapy. These diseases are presently diagnosed and classified by morphological criteria. These criteria include the subjective interpretation of pattern of growth (nodular or diffuse), nuclear size and configuration, amount of cytoplasm, and relative numbers of small and large cells (18). Despite ongoing efforts to refine morphological criteria, pathologists are still unable to accurately predict the behavior of many of these tumors. In addition, difficulties in interpretation have resulted in a suboptimal degree of reproducibility of histological diagnoses (8). Furthermore, there are a number of cases which cannot be precisely classified on the basis of morphology alone. These difficulties in the morphological assessment of non-Hodgkin's lymphomas have hampered interpretation of the results of clinical therapeutic trials. It is clear, then, that the addition of quantitative and reproducible parameters may aid in the diagnosis and classification of malignant lymphomas and would be a welcome supplement to morphological interpretation.

Flow analysis of cell size using the Coulter principle and flow microfluorometric analysis of cells stained with fluorescent dyes which bind quantitatively to individual cell constituents have recently been applied to the study of human neoplastic lymphoid cell populations (3—6, 9, 15, 23, 25). Such techniques permit the rapid and accurate quantitation of cellular DNA (3—6, 9, 15, 23, 25), RNA (1, 11), protein content (10), cell surface antigens (17), and other physical and biochemical properties of cells (16). In a previous study of nonneoplastic and neoplastic lymphoid cells, we measured electronic cell size and DNA distributions by FMF (6). A general correlation between the percentage of cells in the S phase of the cell cycle and the expected clinical behavior of lymphomas and lymphoid leukemias was found. In that study, a correlation between the cell size observed morphologically and the electronic cell size was also demonstrated.

Several recent studies utilizing both static and flow microfluorometric methods for the determination of DNA content in the non-Hodgkin's lymphomas have shown that many malignant lymphomas have measurable abnormalities in DNA content (3—5, 22—25). Since karyotype abnormalities have not only been shown to be associated with malignancy (21) but may also have prognostic significance in the leukemias (13), DNA content abnormalities may have a similar significance in hematopoietic neoplasia. Cell cycle kinetic data available from flow analysis of DNA content may prove to be a valuable predictor of tumor behavior as well. In this study, we compare Coulter volume measurements, light scatter determinations, and DNA histograms obtained by flow analysis from 30 non-Hodgkin's lymphomas with those obtained from 31 nonneoplastic lymphoid populations. This approach was undertaken to assess these parameters as a quantitative and reproducible means of evaluating non-Hodgkin's lymphoma.

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4 The abbreviations used are: FMF, flow microfluorometry; PHA, phytohemagglutinin; MV, modal volume; CEN, chicken erythrocyte nuclei; CV, coefficient of variation.
MATERIALS AND METHODS

Patients and Controls. Neoplastic samples from patients with non-Hodgkin's lymphomas were obtained from solid lymphoid tissue (lymph node, spleen, and tumor masses) immediately following surgery in 28 cases. In 2 patients, malignant cells were obtained from pleural effusions. No patient was undergoing antineoplastic therapy at the time of study. In several cases, however, the material studied was taken at the time of relapse of the malignant lymphoma. Controls were obtained in a similar manner from nonneoplastic lymphoid tissue (tonsils, lymph nodes, spleen, and peripheral blood) in 26 patients. Additional controls consisted of normal blood mononuclear cells obtained from 5 volunteers. In 3 cases, the normal peripheral blood cells were studied following incubation with PHA (Difco Laboratories, Detroit, Mich.) at a concentration of 10 μg/10⁶ cells/ml in the presence of 5% heat-treated pooled human serum for 72 hr at 37°C. In all samples obtained for diagnosis, routine histological sections or cytological preparations were available for conventional morphological evaluation. The non-Hodgkin's lymphomas were classified according to a modification of the scheme of Rappaport (18, 19).

Preparation of Cell Suspensions. Solid tissue was finely minced in Roswell Park Memorial Institute Culture Medium 1640 (Grand Island Biological Co., Grand Island, N. Y.) and then filtered through a stainless steel mesh. Unless otherwise specified, all procedures were carried out at room temperature. Peripheral blood samples, tonsils, pleural effusions, and splenic suspensions were diluted with medium and centrifuged through a Ficoll-sodium diatrizoate mixture (Bionetics, Kensington, Md.) for 30 min at 400 × g. Prior to washing, contaminating RBC were lysed with 0.83% ammonium chloride as necessary. In one case, the mononuclear cells were immediately fixed without washing in filtered 50% ethanol. In all other cases, the cells were washed twice in medium prior to fixation. The viabilities of the final cell suspensions were tested by trypan blue exclusion. The viability was greater than 70% in all cases except one in which the viability was 65%. Smears were prepared from each cell suspension and stained with a modified Wright stain (Diff-Quik; Harleco, Gibbstown, N. J.) for morphological evaluation. All samples were fixed in filtered 50% ethanol and stored at 4°C for up to 12 months prior to study by FMF.

Electronic Cell Volume Determinations. Cell volume frequency distributions were obtained on unfixed cells in 49 cases with a Coulter Counter Model ZBI (70-μm-diameter orifice) interfaced with a 100-channel pulse height analyzer and an X-Y plotter (Coulter Electronics, Inc., Hialeah, Fla.). The system was calibrated using 10.12-μm polystyrene spheres. The MV (the electronic cell volume corresponding to the peak channel) was calculated in each case. All frequency distributions contained 4000 cells in the peak channel.

Preparation of Internal Biological Standard for FMF. Nuclei isolated from chicken erythrocytes, used as an internal standard for FMF, were prepared as follows. Five ml of heparinized chicken blood were diluted in 150 ml of a 0.9% NaCl solution containing 3 mM CaCl₂ and then centrifuged at 400 × g for 15 min. After removing the supernatant, the pellet was resuspended in 10 volumes of saponin (50 mg/100 ml of the CaCl₂ containing 0.9% NaCl solution) and agitated for 10 min. The nuclei were then washed 3 times in the CaCl₂-containing 0.9% NaCl solution, fixed in filtered 50% ethanol, and stored at 4°C. Nuclei isolated from several chickens were shown to have identical DNA content when mixed together and stained with propidium iodide. In addition, nuclei isolated from the same chicken had a stable DNA content for at least 4 months.

Flow Analysis of DNA Content and Light Scatter. In each case, 1.2 × 10⁸ ethanol-fixed CEN were added to 4 × 10⁶ ethanol-fixed human lymphoid cells, and the mixture was centrifuged at 250 × g for 7 min. The supernatant was aspirated, and the pellet was resuspended in 0.5 ml of RNase (RASE, 4909 units/ml; Worthington Biochemical, Freehold, N. J.) and incubated for 30 min at 37°C. Then, 0.5 ml of propidium iodide [5 mg/100 ml in 1.12% sodium citrate (Calbiochem, San Diego, Calif.)] were added, and the cells were allowed to stain for at least 30 min. The stained cell suspensions were filtered through a 44-μm nylon mesh (Small Parts, Inc., Miami, Fla.) just prior to FMF.

A FACS-II flow microfluorometer equipped with a 50-μm-diameter nozzle (Becton-Dickinson FACS Systems, Mountain View, Calif.) was used for DNA content and light scatter measurements. A 488-nm argon ion laser line at 400 milliwatts was used for excitation, and the total fluorescent emission above 620 nm was measured. An ND-100 pulse height analyzer (Nuclear Data Inc., Schaumburg, Ill.) was used to obtain 256-channel DNA and forward low-angle light scatter histograms. At least 70,000 cells were collected in each DNA histogram. Light scatter histograms of at least 50,000 cells were collected in 51 cases. Doublets of CEN were 'gated out' of the light scatter histograms on the basis of their DNA content. The CV (S.D. × 100/mean channel number) of fluorescence of the CEN was maintained below 5% in all cases. The DNA and light scatter histograms were recorded on magnetic tape. The histograms were then communicated to the Northeast Regional Data Center at the University of Florida and analysis was performed on an Amdahl 470 computer.

The DNA content (degree of ploidy) of the human lymphoid populations is expressed as the ratio between the mean channel number of the human G₀–G₁ peak and the mean channel number of the CEN peak. The means of the CEN and human G₀–G₁ peaks were calculated from the DNA histograms as follows. The S.D. of each of the peaks was estimated from the calculation of the CV at half-height (6), and then the weighted mean was taken over the range of the curve, including 3 S.D. on either side of the mode of the respective peaks. In those lymphomas having bimodal G₀–G₁ distributions with partially overlapping peaks, the DNA ratio was calculated using the mode rather than the mean of the aneuploid peak. In 30 cases, sufficient cells were present for repeat DNA analysis. There was excellent agreement between duplicate DNA ratios in those cases in which repeat values were obtained. The average difference between duplicate DNA ratio values was 0.96%. In cases which were measured in duplicate, the data listed in Tables 1 to 3 represent the values corresponding to the sample with the lower CV of the human G₀–G₁ peak.

Flow microfluorometric analysis of lymphoid cells treated with RNase and then stained with propidium iodide yields a histogram representing the frequency distribution of cellular DNA (10). The largest population in the DNA histogram contains cells with the diploid (2n) amount of DNA. This subpopulation is composed of cells either in the resting phase (G₀) or in the G₁ phase of the cell cycle. A smaller population of cells contains...
twice the amount of DNA (4n) and corresponds for the most part to cells in the G2 phase or in mitosis. Cells undergoing DNA synthesis (S) contain quantities of DNA which are intermediate between 2n and 4n and therefore fall between these 2 populations. Noncycling cells flowing as doublets and passing simultaneously through the laser beam would display 4n amounts of DNA and would be interpreted as cells in G2 or mitosis. Therefore, we chose to analyze only the percentage of cells with S-phase amount of DNA, which should not be significantly affected by the presence of doublets. The percentage of cells in the S phase of the cell cycle was determined according to Model 1 of the methods of Baisch et al. (2). In those malignant lymphomas with bimodal G0-G1, distributions and overlapping DNA content between presumably nonneoplastic and neoplastic cells, no attempt was made to calculate the percentage of cells in the S phase of the cell cycle.

In this study, the normal ranges for DNA, light scatter, and Coulter measurements are defined as the mean ± 2 S.D. of the values obtained from the 31 morphologically nonneoplastic controls. In order to be sure that differences in the CV of the G0-G1 peak of the DNA histogram between nonneoplastic and neoplastic cases were not due to minor differences in instrument setting, a corrected CV value was calculated for all cases using the formula:

\[ \text{Corrected CV} = \frac{\text{Observed CV} \times R}{R + K(1 - R)} \]

where \( R = 5.0/\text{CV of CEN} \) and \( K = 1/\text{DNA ratio} \). This correction takes into account the differences in the CV of the control CEN data to standardize the CV value to 5%. Using this formula, the same linear transformation is applied to the human data, thereby correcting the CV values of the human peak for a CEN CV of 5%. The corrected CV values were used to define the normal range for CV of the G0-G1 peak.

The cell size as determined by Coulter volume measurements on unfixed cells is expressed as the ratio of the MV of the human lymphoid cells to the MV of unfixed CEN measured separately (Tables 1 to 3). A similar ratio between the mode (peak channel) of the human population and the mode of the CEN internal standard is used to express the light scatter measurements of the ethanol-fixed propidium iodide-stained cells. In cases with bimodal cell size distributions, the Coulter and light scatter ratios were determined using the peak with the higher MV or light scatter intensity, respectively. To determine the usefulness of cell size alone in discriminating between nonneoplastic lymphoid populations and lymphoid populations obtained from non-Hodgkin's lymphomas, the Coulter volume and light scatter ratios of the neoplastic cases were compared with the corresponding ratios from the lymphoid populations obtained from solid tissue.

For purposes of easier comparison between neoplastic cases, the non-Hodgkin's lymphomas were divided into 2 categories, "low-grade" and "high-grade," according to their expected clinical behavior. In this study, low-grade lymphomas include: well-differentiated lymphocytic lymphoma; lymphocytic lymphoma, intermediate differentiation; nodular poorly differentiated lymphocytic lymphoma; and nodular lymphoma, mixed cell type. High-grade lymphomas include: lymphoblastic lymphoma; diffuse lymphoma, mixed cell type; diffuse "histiocytic" lymphoma; diffuse poorly differentiated lymphocytic lymphoma; and immunoblastic lymphoma.

**RESULTS**

**DNA Content.** The means for the DNA ratio, CV, and S-phase values of the 31 nonneoplastic controls are listed in Table 1 by specimen type. These controls encompassed a wide range of morphologically normal and "reactive" lymphoid cell populations. These cases included mononuclear cells isolated from: normal peripheral blood; PHA-stimulated peripheral blood; peripheral blood from patients with infectious mononucleosis; hyperplastic tonsils; normal spleen; spleens with lymphoid hyperplasia; and several lymph nodes with non-specific follicular hyperplasia, diffuse hyperplasia, or granulomatous lymphadenitis. The mean ± S.D. of the DNA ratios of the 31 nonneoplastic cases was 2.83 ± 0.08. The mean ± S.D. of the corrected CV values in these cases was 3.31 ± 0.30%.

All of the nonneoplastic cases had unimodal G0-G1, peaks (Chart 1). The CV values ranged from 2.27 to 3.63% (mean, 3.09 ± 0.32%). The S-phase values varied from 0.6 to 11.2%.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Diagnosis</th>
<th>No. of cases</th>
<th>DNA ratio</th>
<th>CV (%)</th>
<th>S (%)</th>
<th>Coulter ratio</th>
<th>Light scatter ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peripheral blood</td>
<td>Normal</td>
<td>2</td>
<td>2.82 ± 0.12</td>
<td>3.32 ± 0.40</td>
<td>1.00 ± 0.57</td>
<td>9.39 ± 0.0</td>
<td>3.89 ± 0.33</td>
</tr>
<tr>
<td>Peripheral blood</td>
<td>Infectious</td>
<td>3</td>
<td>2.83 ± 0.10</td>
<td>2.62 ± 0.31</td>
<td>4.43 ± 2.37</td>
<td>10.27 ± 1.72</td>
<td>3.49 ± 0.54</td>
</tr>
<tr>
<td>Peripheral blood</td>
<td>mononucleosis</td>
<td>3</td>
<td>2.74 ± 0.05</td>
<td>3.28 ± 0.25</td>
<td>6.83 ± 3.78</td>
<td>9.90 ± 1.11</td>
<td>4.38 ± 0.20</td>
</tr>
<tr>
<td>Lymph node and</td>
<td>PHA-stimulated</td>
<td>3</td>
<td>2.86 ± 0.08</td>
<td>3.08 ± 0.29</td>
<td>4.77 ± 2.56</td>
<td>7.61 ± 0.78</td>
<td>3.06 ± 0.52</td>
</tr>
<tr>
<td>tonsil</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spleen</td>
<td>Nonneoplastic</td>
<td>5</td>
<td>2.81 ± 0.02</td>
<td>3.22 ± 0.22</td>
<td>3.82 ± 1.81</td>
<td>8.63 ± 0.93</td>
<td>3.69 ± 1.10</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>31</td>
<td>2.83 ± 0.08</td>
<td>3.09 ± 0.32</td>
<td>4.5 ± 2.6</td>
<td>7.85 ± 0.91</td>
<td>3.23 ± 0.74</td>
</tr>
</tbody>
</table>

| a | Ratio between mean of human G0-G1, peak and mean of CEN peak. |
| b | CV of the human G0-G1, peak. |
| c | Cells in the S area of the human DNA distribution. |
| d | Ratio between modal volume of human cells and modal volume of CEN (unfixed cells). |
| e | Ratio between peak channel of human cells and peak channel of CEN. |
| f | Mean ± S.D. |
| g | Mean value for 16 cases. |
| h | Mean value for 14 cases. |
| i | Mean value for solid tissues only (21 cases). |
| j | Mean value for solid tissues only (19 cases). |
The lowest S-phase values occurred in normal peripheral blood, and the highest S-phase values were seen in tonsils with marked follicular hyperplasia and PHA-stimulated peripheral blood.

The DNA ratio, CV, and S-phase values of the 15 low-grade and 15 high-grade lymphomas are listed by diagnosis in Tables 2 and 3, respectively. Eight of 15 low-grade and 7 of 15 high-grade lymphomas had unimodal G0-G1 peaks with DNA ratios in the normal diploid range. Of these 15 cases with normal DNA ratios, 4 low-grade lymphomas (Table 2, Cases 2, 6, 9, and 10) and 2 high-grade lymphomas (Table 3, Cases 5 and 15) could be distinguished from the nonneoplastic controls on the basis of their corrected CV values. One low-grade lymphoma and 3 high-grade lymphomas had bimodal G0-G1 distributions with one population of cells having a DNA ratio in the normal diploid range and one population of cells having a hyperdiploid DNA ratio (Chart 2). One low-grade case showed, in addition to diploid cells, a predominant tetraploid population from which the DNA ratio was calculated. One high-grade lymphoma had a bimodal G0-G1 distribution with diploid cells mixed with hypodiploid tumor cells. In one case of lymphoblastic lymphoma (high-grade), there were 2 distinct populations of tumor cells on the basis of DNA content, each with a markedly hyperdiploid DNA ratio, as well as G0-G1 cells with a diploid DNA ratio (Chart 3). In 5 low-grade and 3 high-grade lymphomas, the tumor cells had unimodal G0-G1 distributions with hyperdiploid DNA ratios. Five of these 8 cases had widened

### Table 2

Flow analysis of low-grade neoplasms

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Diagnosis</th>
<th>DNA ratio</th>
<th>CV (%)</th>
<th>S (%)</th>
<th>Coulter ratio</th>
<th>Light scatter ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Spleen</td>
<td>Well-differentiated lymphocytic lymphoma</td>
<td>3.28</td>
<td>5.44</td>
<td>7.0</td>
<td>12.17</td>
<td>2.96</td>
</tr>
<tr>
<td>2. Spleen</td>
<td>Well-differentiated lymphocytic lymphoma</td>
<td>2.73</td>
<td>3.67</td>
<td>3.0</td>
<td>6.96</td>
<td>3.12</td>
</tr>
<tr>
<td>3. Spleen</td>
<td>Well-differentiated lymphocytic lymphoma</td>
<td>2.85</td>
<td>3.09</td>
<td>3.9</td>
<td>9.31</td>
<td>3.96</td>
</tr>
<tr>
<td>4. Lymph node</td>
<td>Well-differentiated lymphocytic lymphoma</td>
<td>2.84</td>
<td>2.83</td>
<td>2.9</td>
<td>10.09</td>
<td>ND</td>
</tr>
<tr>
<td>5. Lymph node</td>
<td>Lymphocytic lymphoma intermediate differentiation</td>
<td>3.32</td>
<td>3.65</td>
<td>2.7</td>
<td>9.39</td>
<td>2.56</td>
</tr>
<tr>
<td>6. Lymph node</td>
<td>Nodular poorly differentiated lymphocytic lymphoma</td>
<td>2.91</td>
<td>3.84</td>
<td>3.6</td>
<td>9.39</td>
<td>2.54</td>
</tr>
<tr>
<td>7. Lymph node</td>
<td>Nodular poorly differentiated lymphocytic lymphoma</td>
<td>3.23</td>
<td>4.28</td>
<td>6.7</td>
<td>12.87</td>
<td>3.24</td>
</tr>
<tr>
<td>8. Lymph node</td>
<td>Nodular poorly differentiated lymphocytic lymphoma</td>
<td>2.74</td>
<td>3.17</td>
<td>1.5</td>
<td>ND</td>
<td>3.54</td>
</tr>
<tr>
<td>9. Lymph node</td>
<td>Nodular poorly differentiated lymphocytic lymphoma</td>
<td>2.91</td>
<td>3.86</td>
<td>4.3</td>
<td>7.31</td>
<td>2.76</td>
</tr>
<tr>
<td>10. Lymph node</td>
<td>Nodular poorly differentiated lymphocytic lymphoma</td>
<td>2.91</td>
<td>3.83</td>
<td>3.1</td>
<td>7.31</td>
<td>3.16</td>
</tr>
<tr>
<td>11. Lymph node</td>
<td>Nodular poorly differentiated lymphocytic lymphoma</td>
<td>2.97</td>
<td>2.84</td>
<td>2.0</td>
<td>7.31</td>
<td>4.82</td>
</tr>
<tr>
<td>12. Lymph node</td>
<td>Nodular poorly differentiated lymphocytic lymphoma</td>
<td>3.01</td>
<td>3.48</td>
<td>2.2</td>
<td>10.09</td>
<td>4.83</td>
</tr>
<tr>
<td>13. Lymph node</td>
<td>Nodular poorly differentiated lymphocytic lymphoma</td>
<td>3.05</td>
<td>5.28</td>
<td>4.8</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>14. Lymph node</td>
<td>Nodular lymphoma mixed cell type</td>
<td>3.20</td>
<td>NC</td>
<td>NC</td>
<td>11.83</td>
<td>3.04</td>
</tr>
<tr>
<td>15. Lymph node</td>
<td>Nodular lymphoma mixed cell type</td>
<td>6.02</td>
<td>3.25</td>
<td>NS</td>
<td>21.57</td>
<td>4.38</td>
</tr>
</tbody>
</table>

- a Ratio between mean of human G0-G1 peak and mean of CEN peak.
- b CV of the human G0-G1 peak.
- c Cells in the S area of the human DNA distribution.
- d Ratio between modal volume of human cells and modal volume of CEN (unfixed cells).
- e Ratio between peak channel of human cells and peak channel of CEN.
- f ND, not done; NC, not calculated due to bimodal partially overlapping G0-G1 populations.
- g Bimodal G0-G1 population with partially overlapping peaks. DNA ratio calculated for abnormal mode.
- h DNA ratio calculated from tetraploid population.
- i No distinct S-phase population seen beyond tetraploid peak.
- j Bimodal distribution. Ratio calculated from larger peak.
Flow analysis of high-grade neoplasms

**Table 3**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Diagnosis</th>
<th>DNA ratio&lt;sup&gt;a&lt;/sup&gt;</th>
<th>CV&lt;sup&gt;b&lt;/sup&gt; (%)</th>
<th>Coulter ratio&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Light scatter ratio&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Lymph node</td>
<td>Lymphoblastic lymphoma</td>
<td>5.00/5.69</td>
<td>NC&lt;sup&gt;e&lt;/sup&gt;</td>
<td>NC</td>
<td>23.30</td>
</tr>
<tr>
<td>2. Pleural fluid</td>
<td>Lymphoblastic lymphoma</td>
<td>2.75</td>
<td>3.35</td>
<td>4.7</td>
<td>11.13</td>
</tr>
<tr>
<td>3. Lymph node</td>
<td>Diffuse poorly differentiated lymphocytic lymphoma</td>
<td>3.28</td>
<td>NC&lt;sup&gt;e&lt;/sup&gt;</td>
<td>NC</td>
<td>ND</td>
</tr>
<tr>
<td>4. Lymph node</td>
<td>Diffuse lymphoma mixed cell type</td>
<td>2.77</td>
<td>3.02</td>
<td>6.0</td>
<td>10.76</td>
</tr>
<tr>
<td>5. Lymph node</td>
<td>Diffuse lymphoma mixed cell type</td>
<td>2.92</td>
<td>3.78</td>
<td>4.5</td>
<td>ND</td>
</tr>
<tr>
<td>6. Lymph node</td>
<td>Diffuse histiocytic lymphoma</td>
<td>2.62</td>
<td>4.89</td>
<td>NC</td>
<td>10.09</td>
</tr>
<tr>
<td>7. Lymph node</td>
<td>Immunoblastic lymphoma</td>
<td>2.90</td>
<td>3.16</td>
<td>8.3</td>
<td>9.05</td>
</tr>
<tr>
<td>8. s.c. tissue</td>
<td>Diffuse histiocytic lymphoma</td>
<td>3.32</td>
<td>4.51</td>
<td>11.3</td>
<td>28.53</td>
</tr>
<tr>
<td>10. Pleural fluid</td>
<td>Diffuse histiocytic lymphoma</td>
<td>4.52</td>
<td>3.72</td>
<td>NC</td>
<td>29.22</td>
</tr>
<tr>
<td>11. Lymph node</td>
<td>Diffuse histiocytic lymphoma</td>
<td>5.23</td>
<td>22.14</td>
<td>NC</td>
<td>ND</td>
</tr>
<tr>
<td>12. Lymph node</td>
<td>Diffuse histiocytic lymphoma</td>
<td>2.87</td>
<td>3.54</td>
<td>4.6</td>
<td>ND</td>
</tr>
<tr>
<td>13. Lymph node</td>
<td>Diffuse histiocytic lymphoma</td>
<td>3.13</td>
<td>4.55</td>
<td>21.6</td>
<td>ND</td>
</tr>
<tr>
<td>14. Lymph node</td>
<td>Diffuse histiocytic lymphoma</td>
<td>3.25</td>
<td>3.50</td>
<td>9.7</td>
<td>ND</td>
</tr>
<tr>
<td>15. Lymph node</td>
<td>Diffuse histiocytic lymphoma</td>
<td>2.96</td>
<td>4.15</td>
<td>9.8</td>
<td>ND</td>
</tr>
</tbody>
</table>

<sup>a</sup> Ratio between mean of human G0—G1 peak and mean of CEN peak.

<sup>b</sup> CV of the human G0—G1 peak.

<sup>c</sup> Cells in the S area of the human DNA distribution.

<sup>d</sup> Ratio between modal volume of human cells and modal volume of CEN (unfixed cells).

<sup>e</sup> Ratio between peak channel of human cells and peak channel of CEN.

<sup>f</sup> Two distinct hyperdiploid peaks observed.

<sup>g</sup> NC, not calculated due to bimodal partially overlapping G0—G1 populations; ND, not done.

<sup>h</sup> Bimodal distribution. Ratio calculated from larger peak.

<sup>i</sup> Bimodal G0—G1, population with partially overlapping peaks. DNA ratio calculated for abnormal mode.

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**Chart 2.** DNA distribution obtained from a case of nodular lymphoma mixed cell type. Channel number represents relative fluorescence intensity (DNA). The CEN fall in Channel 25. The peak in Channel 50 represents doublets of CEN. A bimodal human G0—G1 distribution is seen; the peak to the left has a normal DNA ratio and presumably represents nonneoplastic elements; the peak to the right has a hyperdiploid DNA ratio.

**Chart 3.** DNA distribution obtained from a case of lymphoblastic lymphoma. Channel number represents relative fluorescence intensity (DNA). Singlets and doublets of CEN fall in Channels 25 and 50, respectively. A human diploid population is present. In addition, 2 distinct hyperdiploid peaks are seen.

G0—G1 peaks with CV values ranging from 4.28 to 5.44%. In one case (Table 2, Case 5) with a unimodal hyperdiploid tumor cell population, mixing experiments with equal numbers of nonneoplastic diploid cells obtained from normal peripheral blood confirmed the hyperdiploid DNA content within the tumor cells (Chart 4). The DNA ratios for all 61 cases studied are summarized in Chart 5 according to morphological diagnosis.

The S-phase values, in those neoplastic cases in which it could be calculated, varied from 1.5 to 21.6%. Eleven of 13 low-grade lymphomas had S-phase values of less than 5% (Chart 6). The 2 low-grade lymphomas with S-phase values greater than 5% had abnormal DNA ratios. Seven of 10 high-grade lymphomas had S-phase values greater than 5% (Chart 6). All 3 of the high-grade lymphomas with S-phase values under 5% had normal DNA ratios.

**Coulter Volume and Light Scatter Measurements.** All of the nonneoplastic controls (obtained from solid tissue) had unimodal cell size distributions by both Coulter volume and light scatter determinations. The Coulter volume ratios in the 21
nonneoplastic cases ranged from 6.61 to 10.09 (mean, 7.85 ± 0.91). The light scatter ratios from 19 cases ranged from 2.25 to 5.08 (mean, 3.23 ± 0.74). Six of 13 low-grade lymphomas and 7 of 8 high-grade lymphomas in which Coulter volume determinations were performed could be differentiated from the nonneoplastic cases on the basis of Coulter volume ratios (Chart 7). Only one of 13 low-grade lymphomas and 5 of 12 high-grade lymphomas could be distinguished from the control populations on the basis of light scatter ratios (Chart 8). Four of the neoplastic cases with normal DNA content (on the basis of both the DNA ratio and the CV of the G0-G1 peak) were abnormal with respect to Coulter volume ratio. One neoplastic case with normal DNA content in which the Coulter volume was not determined had an abnormal light scatter ratio.

The immunoblastic lymphoma arose in the background of angioimmunoblastic lymphadenopathy, and the morphological diagnosis of malignant lymphoma was based on the finding of clusters of large atypical "immunoblasts" intermingled with small lymphocytes and plasma cells (19). Although the Coulter volume and light scatter ratios were within the normal range in this case, both the Coulter volume distribution and the light scatter distribution appeared distinctly different from the corresponding cell size patterns obtained from reactive lymphoid populations. By both Coulter volume (not shown) and light scatter (Chart 9), there was a significant tail in the descending portion of the histograms that was not observed in nonneoplastic lymphoid populations in our series (Chart 10). Obviously, this population of larger cells is not reflected in the calculation of cell size ratios based only on the peak channel of the human lymphoid cells.

A linear regression analysis was performed comparing Coulter volume ratios with light scatter ratios in the 46 cases in
Flow Analysis of Non-Hodgkin’s Lymphoma

Chart 7. Coulter volume ratios of low-grade (•) and high-grade (○) neoplasms. Bar, mean ± 2 S.D. of Coulter volume ratios obtained from 21 nonneoplastic lymphoid tissues.

Chart 8. Light scatter ratios of low-grade (•) and high-grade (○) neoplasms. Bar, mean ± 2 S.D. of light scatter ratios obtained from 19 nonneoplastic lymphoid tissues.

Chart 9. Light scatter distribution from a case of immunoblastic lymphoma. Channel number represents relative light scatter intensity. The first peak corresponds to CEN. A significant population of cells with large light scatter intensity is present beyond Channel 130.

Chart 10. Representative light scatter distribution from a nonneoplastic lymphoid population. Channel number represents relative light scatter intensity. The first peak corresponds to CEN. The human population contains relatively few cells beyond Channel 130.

which both determinations were made. The correlation coefficient was 0.63.

DISCUSSION

Hematopathologists are currently giving considerable attention to the problem of providing a reproducible and clinically useful classification for the non-Hodgkin’s lymphomas (12). There are inherent difficulties, however, in the morphological
 diagonal processes may make differential diagnosis particularly difficult.

The introduction of additional parameters in the assessment of hematological malignancy may be of assistance in both diagnosis and classification of lymphomas and leukemias. Cytogenetic analysis (21) and the characterization of cell surface receptors (7) have been particularly useful in this regard. The measurement of cellular DNA has also been applied to the study of lymphoid neoplasia (3–6, 9, 15, 22–25). Sandritter and Grimm (22), using static methods, found abnormal DNA distributions in 18 of 26 cases of non-Hodgkin’s lymphoma, including all cases diagnosed as histiocytic lymphoma or lymphoblastic lymphoma. The nodular lymphomas and the small cell diffuse lymphomas tended to have a diploid DNA content. Using similar techniques, Silvestrini et al. (24) found aneuploid DNA content in several diffuse lymphomas.

Flow methods have also been applied to the study of DNA content in the human lymphomas and leukemias (3–6, 9, 15, 23, 25). In a study of bone marrow cells from 7 patients with lymphoma and 168 patients with leukemia, Barlogie et al. (4) found abnormal DNA distributions in 29 patients for an overall incidence of 16.6%. Concurrent cytogenetic analysis carried out on 160 patients revealed abnormalities in 50% of acute leukemias, indicating that an abnormal cellular DNA content does not necessarily coincide with changes in the number or arrangement of chromosomes. Presumably, aneuploid karyotypes may be the result of abnormalities in DNA packaging rather than an abnormality in total DNA content in some cases (14). Nevertheless, in the study of Barlogie et al. (4), several patients with normal karyotypes had abnormal DNA histograms as determined by flow analysis, suggesting that the mitoses studied were not representative of the neoplastic populations. One of the advantages of flow analysis of cellular DNA content is that it is not dependent on cellular proliferation.

In a recent study of cellular DNA content by flow cytometry in malignant tumors obtained from solid tumors, all 3 malignant lymphomas studied had aneuploid DNA content (3). Of particular interest in that study was the increase in cellular DNA content observed in one case with a change in morphology from angioimmunoblastic lymphadenopathy to immunoblastic lymphoma. In this study, the measurement of DNA content in the human G0–G1 populations was based on the calculation of DNA ratios, using CEN as an internal standard. Other investigators have used granulocytes or lymphocytes obtained from normal human subjects (3, 23) or whole chicken erythrocytes (26) as a reference for diploid DNA content. We found that nuclei isolated from chicken erythrocytes are a reliable internal standard which can be used to calculate the degree of ploidy of human cells. Furthermore, the use of this type of control, which does not overlap with the human population (26), allows us to monitor the performance of the instrument in each experiment as reflected by the CV of the CEN. In this way, meaningful comparisons between the CV’s of the human populations can be made with reference to the CEN.

We observed that nonneoplastic lymphoid populations, including several cases containing a high percentage of large transformed lymphocytes which morphologically resemble neoplastic elements (infectious mononucleosis, PHA-stimulated cells), had DNA ratios which fell within a narrow range. Fifteen of 30 non-Hodgkin’s lymphomas had abnormalities in DNA content. Fourteen of these 15 cases were hyperdiploid, and one case was hypodiploid. Seven of these cases contained a diploid population, presumably nonneoplastic, in addition to the aneuploid neoplastic cells. In 8 cases, no diploid elements were seen. In order to compare our method of evaluating the degree of ploidy with the methods of other investigators using human diploid cells as a DNA reference (3), we mixed normal human mononuclear cells with the cells of one of these 8 cases. The DNA histogram of the mixture showed 2 partially overlapping populations confirming the aneuploid DNA content of the neoplastic cells. One case of lymphoblastic lymphoma contained 3 populations of cells on the basis of DNA content. One population fell in the normal diploid range, and 2 populations of cells had differing DNA contents which were markedly hyperdiploid. The significance of this finding is unclear, but a similar observation in a case of multiple myeloma having 3 aneuploid populations has recently been made (3).

In 6 cases with normal DNA ratios, the CV of the G0–G1 peak, corrected for differences in instrument settings, was greater than that seen in the nonneoplastic populations studied thus far. An abnormally wide CV may reflect greater variability in cellular DNA content of neoplastic populations or may represent the summation of 2 populations with slightly different mean DNA contents.

We again observed a correlation between the percentage of cells in the S phase of the cell cycle of malignant lymphomas and the expected clinical behavior of these tumors. Thus, with 2 exceptions, the morphologically low-grade lymphomas, known to have a longer median survival (20), had S-phase values of less than 5%. Interestingly, both of the low-grade lymphomas with S-phase values greater than 5% had aneuploid DNA content, which may indicate a more aggressive behavior. Higher S-phase values were observed in high-grade lymphomas, especially in those which morphologically had a significant percentage of large cells. These lymphomas are clinically known to be more aggressive (20). The nonneoplastic lymphoid controls had a wide range of S-phase values. As expected, the highest S-phase values were seen in those nonneoplastic populations with a significant percentage of large cells morphologically, such as PHA-stimulated lymphocytes, hyperplastic tonsils, diffuse lymphoid hyperplasia, and infectious mononucleosis. We did not calculate the percentage of malignant cells in the S phase of the cell cycle in several lymphomas with partially overlapping G0–G1 populations. We feel, at least with regard to the malignant lymphomas, that it is unreasonable to assume that the presumably nonneoplastic diploid cell population does not contribute significantly to the observed S-phase population (3). The S-phase values in this study are slightly higher than those reported previously (6). In this previous study, the percentage of cells in S was calculated manually using a method which underestimates the true percentage of cells in this phase of the cell cycle.

We were able to confirm the previous observation (6) that there is a general correlation between the size distribution of cell populations as observed by optical methods and measured electronically. A number of neoplastic cases were distinguishable from the nonneoplastic controls obtained from solid tissue.
on the basis of the ratio of their Coulter MV to the MV of the CEN. In this study, we compared the modal cell size of lymphoid populations as determined by Coulter volume analysis on unfixed cells to measurements of light scatter on fixed, stained cells. In doing so, we were not attempting to determine absolute cell size by light scatter. We were interested, however, in determining if light scatter measurements could give practical, useful information regarding the relative cell size of different lymphoid populations under our conditions. Although some correlation between Coulter volume measurements and light scatter measurements was observed, fewer malignant lymphomas were distinguishable from our nonneoplastic controls by light scatter than by Coulter volume. We can conclude, however, that at least in some lymphomas which contain both large and small elements morphologically, 2 subpopulations may be distinguishable by light scatter characteristics (Charts 9 and 11).

In this study, we found that an almost equal percentage of low-grade lymphomas and high-grade lymphomas had aneuploid DNA content (47% versus 53%). We did find, however, that high-grade lymphomas are much more likely than low-grade lymphomas to have an S-phase value greater than 5% and that by electronic cell size measurements a higher percentage of high-grade lymphomas could be distinguished from nonneoplastic controls. These findings are not unexpected, since the most common high-grade lymphomas are composed of morphologically large cells.

In our series, 15 of 30 non-Hodgkin’s lymphomas had aneuploid DNA content. Of the remaining 15 cases with normal DNA ratios, 6 additional cases could be distinguished from nonneoplastic controls by their corrected CV, 4 cases differed on the basis of Coulter volume alone, and one case differed by light scatter alone. Altogether, by all 3 flow parameters studied, 26 of 30 (87%) of the neoplastic cases could be distinguished from nonneoplastic cases.

Dual-parameter analysis of DNA content and other cellular parameters such as RNA, protein, cell surface antigens, and light scatter may yield extremely useful information in those lymphomas in which the malignant population is intermixed with residual or reactive elements. The separation of the 2 populations by an independent parameter would allow the accurate determination of the proliferative characteristics of each cell population. Most non-Hodgkin’s lymphomas in adults bear monoclonal surface immunoglobulin (7), making dual staining for cell surface immunoglobulin and DNA particularly attractive in the evaluation of these neoplasms. We have specifically chosen propidium iodide as a DNA stain because of its compatibility with fluorescein as a second fluorochrome in dual-labeling experiments of DNA and surface immunoglobulin. Our use of washed, whole-cell, ethanol-fixed preparations is also based on our intent to perform dual-labeling experiments in the future.

Our studies show that flow analysis does provide rapid, quantitative, and reproducible data about DNA content and cell size which can be of use in the evaluation of the non-Hodgkin’s lymphomas. Since many malignant lymphomas appear to have abnormal DNA content, FMF studies should be of diagnostic value, particularly in distinguishing between active nonneoplastic and neoplastic lymphoid populations. Flow analysis may also be useful in assessing prognosis and monitoring therapy in patients with malignant lymphomas. The study of a larger number of cases with appropriate clinical correlation will be necessary to determine the full value of this technique in the management of these patients.

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Flow Analysis of DNA Content and Cell Size in Non-Hodgkin's Lymphoma

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