Heterogeneity of Non-Hodgkin’s Lymphoma Probed by Nucleic Acid Cytometry

By John Srigley, Bart Barlogie, James J. Butler, Barbara Osborne, Mark Blick, Dennis Johnston, Hagop Kantarjian, James Reuben, John Batsakis, and Emil J Freireich

Flow cytometric analyses of cellular DNA, RNA, and double-stranded RNA content were performed on lymph nodes and extranodal tissue from 177 patients with non-Hodgkin’s lymphoma. With increasing histologic grade, a higher incidence of aneuploidy, higher proliferative activity, and higher total and double-stranded RNA content were found. Despite considerable cytometric heterogeneity within histologic grades and morphologic subtypes, conformity between cytometric and morphologic classifications was observed in 85% of cases. Among intermediate-grade and high-grade lymphomas, increased proliferative activity and diploidy were associated with more frequent responses to treatment. Thus, nucleic acid-derived parameters relate to morphologic subtypes and permit an objective approach to lymphoma classification based on ploidy, proliferation, and RNA characteristics that also had prognostic implications.

The non-Hodgkin’s lymphomas (NHL) represent a spectrum of hematologic malignancies characterized by a highly diverse clinical behavior. Although morphologic approaches have been subject to technical and interpretative problems, prognostically relevant classifications based on growth pattern and cell size have evolved. The international working formulation provides a framework for comparison among these most commonly used systems. Advances in immunology have made possible the phenotypic characterization of NHL in relationship to normal lymphocyte differentiation and maturation. At least two of the current taxonomic schemes emphasize a combined immunologic–morphologic approach, but the independent prognostic relevance of phenotypic characteristics has not been established.

The biologic and clinical heterogeneity of NHL is contributed to by factors other than structural and cell surface characteristics. Included among these are genetic, kinetic, and metabolic features. Several investigative approaches including classical cyto genetics, tritiated thymidine labeling, and DNA flow cytometry (FCM) have been used to explore these biologic variables. With use of appropriate fluorescent dyes, nucleic acid FCM provides objective and quantitative measurements of macromolecules in a rapid and statistically applicable manner. Smaller studies of NHL using DNA cytometry have shown that aggressive lymphomas are associated with more frequent ploidy abnormalities and higher proliferative activity than are indolent lesions.

We have evaluated FCM as a quantitative cytologic tool to measure ploidy and cell cycle distribution, as well as total and double-stranded RNA content, in 177 patients with NHL. Our results indicate a high degree of conformity between certain cytometric and morphologic features. In addition, ploidy and cytokinetic findings correlated with the initial response to treatment and survival.

MATERIALS AND METHODS

Study Population

One hundred eighty-five samples from 177 patients with NHL, including 76 at diagnosis and 109 at relapse (23 with histologic transformation), were studied (Table I). Samples were lymph nodes in 157 cases and extranodal tissue in 28. Representative tissue was obtained at biopsy using frozen-section guidance. In 17 instances, samples were obtained by fine-needle aspiration, using multiple passes. In these instances, histologic material taken within 48 hours of the aspiration was also available. As controls, 27 nonneoplastic lymph node samples were used. The lymphomas were classified according to the International Working Formulation scheme and grouped into low-grade (76 cases), intermediate-grade (90 cases), and high-grade histologies (19 cases).

Lymphocyte Marker Studies

Immunologic classification was available in 104 patients, using monoclonal antibodies against T and B cell antigens as well as heavy- and light-chain antisera for surface immunoglobin analysis as previously described. Immunologic phenotyping was conducted by measuring the fluorescence intensity of the aforementioned reagents with an Ortho Spectrum III flow cytometer (Ortho Instruments, Westwood, Mass). B cell lymphoma was diagnosed when >70% of cells expressed a single heavy-chain class or B-1 positivity or when monotypic light-chain expression was noted ($\lambda/\kappa > 3$ and $\lambda/\kappa > 2$). Similarly, T cell disease was confirmed when >70% of cells expressed at least one monoclonal T cell marker. The exception here was the presence of extensive Leu-1 positivity in small lymphocytic lymphomas of B cell type. In ten instances, frozen section immuno peroxidase studies were performed, using a similar panel of antibodies. The remaining patients were placed in an indeterminate category.
Nucleic Acid Flow Cytometry (NA-FCM)

Samples for NA-FCM analysis were placed in RPMI 1640 growth medium (GIBCO, Grand Island, NY) and immediately refrigerated. The sample preparation and analysis were completed within four hours. Biopsies were minced mechanically with surgical blades and sieves and syringed through progressively smaller needles until a single-cell suspension was available. For needle aspirates, syringing was the sole mechanical procedure. Specimens were then washed in phosphate-buffered saline (PBS) and adjusted to a concentration of \(10^6\) cells per cubic centimeter using a Coulter counter (Coulter Electronics, Hialeah, Fla). Two to \(10^6\) cells were obtained from each sample. When sufficient cells were available, an aliquot was fixed in 70% ethanol and refrigerated at 4°C for high-resolution DNA and double-stranded RNA analysis.

For simultaneous DNA–RNA analysis, cells were stained with acridine orange according to a two-step procedure. FCM analysis was carried out with an ICP-22 mercury-arc based cytometer (Phywe, Goettingen, Germany) using appropriate excitation and emission filters. At least 10,000 cells were measured in 90% of samples; the minimum number was 4,000 cells. For DNA-derived ploidy analysis, samples were mixed with normal diploid peripheral blood lymphocytes obtained after Ficoll-Hypaque gradient separation, and the DNA index (DI) was computed, using the ratio of relative DNA contents of test and reference G0,1 cells. For the analysis of the proliferative compartment, a “boxogram” gating procedure along the G1,S interface was used, and the percentage of cells in the (S + G2M) cell cycle compartments was obtained. For DNA aneuploid populations, the (S + G2M) compartment could be computed in 40% of cases because the populations were separable on the basis of their RNA content. In the remaining cases, aneuploid and diploid were not separable on the basis of RNA features; in these instances, an overall proliferative activity of the mixed population was determined. An RNA index was derived of the G0,1 compartment according to criteria previously established; ie, the ratio of the mean RNA content of the sample G0,1 population and the median RNA content of lymphocyte controls. In aneuploid cases, the RNA index of the DNA-abnormal population was used.

When enough cells were available, aneuploidy as determined by the acridine orange technique was confirmed by high-resolution DNA analysis, using the ethidium-bromide/mithramycin dye combination and an ICP-11 mercury-arc–based cytophotometer (Phywe). Aneuploidy (DI \(\neq\) 1) was observed in 37% of 185 cases. DNA abnormalities clustered in the low-degree hyperdiploid and tetraploid regions. There were only four hypodiploid DNA stemlines and three instances of multiple DNA-abnormal clones. Ploidy, proliferative, and RNA features of patients studied at diagnosis and relapse without histologic transformation were similar when analyzed grade for grade. Patients with histologic transformation had a higher incidence of aneuploidy than de novo intermediate- and high-grade lymphomas (\(P = .04\)), but showed similar proliferative and RNA features. There was no relationship between stage of disease and cytometric parameters when they were analyzed grade for grade.

Samples from extranodal sites had higher (\(S + G2M\)%) values than those from nodal sites, 17.7% compared to 9.4% (\(P < .01\)), but ploidy and RNA features were similar. This proliferative difference reflects the high proportion of intermediate and high-grade lymphomas in extranodal sites. There were no significant cytometric differences between samples obtained by tissue biopsy and fine-needle aspiration.

Cytometry and Histologic Grade

Low-grade lymphomas showed a low frequency of aneuploidy, 17% compared with 52% and 47% for the intermediate- and high-grade lesions, respectively (Table 2). Even though overlap was present, the mean proliferative activity increased progressively from 4.7% for low-grade lymphomas to 12.5% for intermediate-grade, and to 26.2% for high-grade lymphomas (Fig 1). RNA index values differed between low-grade, intermediate-grade, and high-grade lymphomas. The “RNA-proliferation maps” demonstrated differences in the location, dispersion, and axis of the confidence ellipses among histologic grades (Fig 2).

<table>
<thead>
<tr>
<th>Histologic Grade</th>
<th>Status at Biopsy</th>
<th>Stage</th>
<th>Biopsy Site</th>
<th>Biopsy Mode</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Diagnosis</td>
<td>I, II</td>
<td>III, IV</td>
<td>Nodal</td>
</tr>
<tr>
<td>Low</td>
<td>35</td>
<td>41</td>
<td>0</td>
<td>11</td>
</tr>
<tr>
<td>Intermediate</td>
<td>35</td>
<td>33</td>
<td>22</td>
<td>28</td>
</tr>
<tr>
<td>High</td>
<td>6</td>
<td>12</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>Total</td>
<td>76</td>
<td>86</td>
<td>23</td>
<td>42</td>
</tr>
</tbody>
</table>

**Table 1. Cytometry of Malignant Lymphoma: Patient Population**

**Statistical Analyses**

Groups of codified data were compared using parametric (t test, analysis of variance, correlation) and non-parametric procedures (Mann-Whitney, Kruskal-Wallis, chi-square). A discriminant function was used to determine the concordance between morphologic and cytometric data. Survival of subgroups was compared using a proportional hazards method.

**RESULTS**

**General Cytometric Features**

Aneuploidy (DI \(\neq 1\)) was observed in 37% of 185 cases. DNA abnormalities clustered in the low-degree hyperdiploid and tetraploid regions. There were only four hypodiploid DNA stemlines and three instances of multiple DNA-abnormal clones. Ploidy, proliferative, and RNA features of patients studied at diagnosis and relapse without histologic transformation were similar when analyzed grade for grade. Patients with histologic transformation had a higher incidence of aneuploidy than de novo intermediate- and high-grade lymphomas (\(P = .04\)), but showed similar proliferative and RNA features. There was no relationship between stage of disease and cytometric parameters when they were analyzed grade for grade.

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Mean ds-RE correlated well with histologic grade: there was a progressive increment with transition from low- to intermediate- to high-grade lymphomas (Table 2). Although normal lymph node samples were diploid and manifested proliferation and RNA index features that were indistinguishable from low-grade lymphoma, the mean ds-RE values were significantly higher in low-grade lymphoma as compared with benign controls \((P < .01)\). In lymphomas in which aneuploid and diploid cell populations were separable by RNA features, the mean \((S + G_2M)\%\) values of the diploid subpopulation was significantly higher than that of nonneoplastic controls \((9.6\% v 5.6%; P < .01)\).

Cytometric and Specific Histologic Subtypes

Cytometric features of individual histologic subtypes of NHL are detailed in Table 3. Small lymphocytic lymphoma showed a 7% frequency of aneuploidy and low mean proliferative and RNA index features. The frequency of DNA-abnormal stemlines increased with the proportion of large cells in the follicular lymphomas with rates of 21%, 37%, and 64% in small cleaved-, mixed-, and large-cell varieties, respectively. In addition, increments in \((S + G_2M)\%\) and RNA index were noted as the proportion of large cells increased. Follicular and diffuse large-cell lymphomas shared similar aneuploidy rates, but the diffuse lesions had significantly higher \((S + G_2M)\%\) values \((15.2\% v 8.0%; P < .01)\). There were also higher values of \((S + G_2M)\%\) and RNA index in diffuse large- \(v\) diffuse mixed-cell lymphomas (only seven cases), but this difference was not significant. A comparison between follicular and diffuse, small, cleaved-cell lymphomas revealed similar aneuploidy rates \((21\% \text{ and } 17\%, \text{ respectively})\), but markedly higher proliferative activity in the latter type \((4.4\% v 8.8%; P < .05)\). In the high-grade category, all four immunoblastic sarcomas were aneuploid and showed higher mean \((S + G_2M)\%\) and RNA index values than nonimmunoblastic diffuse, large-cell lymphomas. The immunoblastic sarcomas had the highest mean RNA values of any histologic subtype. Lymphoblastic and small, noncleaved (undifferentiated) lymphomas showed aneuploidy rates of 50% and 37%, and similar proliferation and RNA features. The four AIDS-related lymphomas (three small noncleaved and one diffuse large-cell) were all diploid and had very high proliferative and RNA features.

Cytometric Classification of Malignant Lymphoma

By discriminant analysis, employing three acridine orange-derived parameters (aneuploidy, \((S + G_2M)\%\), RNA index), the morphologic grade was correctly predicted in 67% of 152 cases (Table 4). When the same analysis was undertaken in 40 patients with additional information on ds-RE values, the prediction of grade was improved to 85%. The ds-RE parameter alone allowed discrimination among grades in 59% of cases. No low-grade lymphoma was predicted as a high-grade lymphoma or vice versa.

Cytometry and Immunophenotype

Aneuploidy rates for the 71 B cell and six T cell lymphomas were 30% and 33%, respectively. Lymphomas of indeterminate phenotype had a higher frequency of abnormal DNA stemlines \((46\% \text{ of } 27 \text{ cases})\) which was accounted for, at least in part, by a higher proportion \((78\%)\) of intermediate- and high-grade
histologic classes. Proliferation and RNA parameters showed no correlation with surface marker status.

**Prognostic Implications of Cytometric Findings**

Among the intermediate- and high-grade lymphomas, 33 patients studied at diagnosis and 57 at relapse were evaluable for response to chemotherapy (Table 5). High proliferative activity at diagnosis (S + G2M% > 15) was associated with a higher complete remission (CR) rate (77% v 41%; \( P = .06 \)). The presence of aneuploidy was an unfavorable feature for attainment of CR in previously treated patients (9% v 32%; \( P = .03 \)). Multivariate regression analysis for survival identified only prior treatment status and high proliferative activity as independent factors adversely affecting survival time.

**DISCUSSION**

We have demonstrated in a group of 177 patients with NHL that conventional morphologic subgroups have distinctly different ploidy and proliferative characteristics as assessed by DNA flow cytometry. This observation is in accord with previous smaller studies using other DNA fluorochromes.\(^{20,21,25,29}\) The strong correlation between proliferative activity and histologic grade in NHL is also in keeping with S-phase labeling studies using tritiated thymidine.\(^{16,17}\) The total RNA content as determined by acridine orange red fluorescence also correlates with histologic grade, but was less discriminatory than the proliferative features. RNA content has been shown in experimental systems to reflect proliferation and cell size, both of which are interrelated.\(^{22,41,42}\) The results of simultaneous DNA and RNA analyses reported herein resemble cytometric observations by other investigators using either light-scatter or Coulter volume analysis coupled with DNA.\(^{20,21,23,25,29}\) In a recent investigation of 220 cases of NHL, Shackney et al noted differences in the relationship of proliferative activity (percentage of cells in S) to Coulter volume among the different lymphoma subgroups.\(^{43}\) Although they are not identical to our "RNA-proliferation maps" (Fig 2), Coulter volume increments seem to correspond to increases in RNA content with progression to more advanced histologic grades. Correlated FCM analysis of DNA, RNA, and cell size should elucidate the independent contributions of these parameters.

In addition, we observed a strong correlation between ds-RE and histologic grade. Although total RNA content and proliferative activity do not allow
diffuse large-cell lymphomas had similar aneuploidy sively from small- to large-cell types. Follicular and activity, and RNA index values increased progres-

There is an association of ds-RNA with tumor viruses, and excess ds-RNA may suggest the patho-

discrimination between low-grade lymphoma and reactive lymphadenopathy, ds-RE values showed sig-

Table 4. Grade Prediction Using Acridine-Orange-Derived Parameters in a Discriminant Function Model

<table>
<thead>
<tr>
<th>Actual Grade</th>
<th>No. of Patients</th>
<th>Predicted Grade Membership</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L</td>
<td>I</td>
</tr>
<tr>
<td>(A) Without ds-RNA excess (n = 152)</td>
<td>66</td>
<td>55</td>
</tr>
<tr>
<td>L*</td>
<td>73</td>
<td>27</td>
</tr>
<tr>
<td>H</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>Correct grade prediction, 67%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(B) With ds-RNA excess (n = 40)</td>
<td>20</td>
<td>19</td>
</tr>
<tr>
<td>L</td>
<td>18</td>
<td>5</td>
</tr>
<tr>
<td>H</td>
<td>2</td>
<td>0</td>
</tr>
</tbody>
</table>
| Correct grade prediction, 85%

*L, low-grade; I, intermediate-grade; H, high-grade histology.

There is little information concerning the relationship of nucleic acid cytometry and immunophenotypes in malignant lymphomas. Contrary to observations by Shackney et al, we did not find a lower aneuploidy rate in T cell disease as compared with B cell disease. The “normal” tissue response in lymph nodes of patients with malignant lymphomas is poorly understood. In those cases in which aneuploid and diploid populations could be resolved on the basis of RNA content, the proliferative activity of the diploid sub-
population was higher than in reactive nodes. This finding suggests a hyperproliferative response to the presence of lymphoma in the alleged normal subpopulation. Although studies addressing this issue are already feasible in lesions with DNA-aneuploid stemlines, alternative neoplastic markers such as nucleolar antigen must be identified for study of the remaining diploid cases. Recent technical advances in flow cytometry and biology should make possible a compre-

Specific morphologic subtypes also showed strong correlation with cytometric parameters. For instance, in follicular lymphoma, aneuploidy rate, proliferative activity, and RNA index values increased progressively from small- to large-cell types. Follicular and diffuse large-cell lymphomas had similar aneuploidy rates, but the latter group had significantly higher proliferative activity, which was also true for the comparison of nodular and diffuse small, cleaved-cell lymphoma. In the high-grade category, the immuno-

Table 5. DNA-Derived Parameters and Remission Induction in Aggressive Lymphoma

<table>
<thead>
<tr>
<th>Cytometric Features</th>
<th>No. of Patients</th>
<th>CR (%)</th>
<th>P Value</th>
<th>No. of Patients</th>
<th>CR (%)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diploid</td>
<td>15</td>
<td>60</td>
<td>0.37</td>
<td>34</td>
<td>32</td>
<td>0.03</td>
</tr>
<tr>
<td>Aneuploid</td>
<td>19</td>
<td>42</td>
<td></td>
<td>23</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>(S + G2M) ≤15%</td>
<td>24</td>
<td>41</td>
<td>0.06</td>
<td>35</td>
<td>26</td>
<td>0.56</td>
</tr>
<tr>
<td>(S + G2M) &gt;15%</td>
<td>9</td>
<td>78</td>
<td></td>
<td>21</td>
<td>19</td>
<td></td>
</tr>
</tbody>
</table>

Data include intermediate- and high-grade histologies. CR, complete remission.
histologic transformation. The finding of comparable FCM results in lymph node biopsy and aspirate material suggests that serial fine-needle aspirations may provide representative material for such studies of clonal evolution and/or phenotypic change during a patient's disease course.

Of equal importance to the differences in cytometric parameters among histologic grades is our observation of considerable heterogeneity within morphologic and immunologic categories of malignant lymphoma. Although the variable natural history of malignant lymphoma has been accounted for in part by morphologic differences, the diversity in nucleic acid features may provide more objective and quantitative criteria for the assessment of prognosis of patients with NHL. Thus, proliferation and ds-RNA excess were the most important features associated with histologic variability, whereas the sensitivity to chemotherapy seemed to be affected by both cytokinetic and ploidy patterns. Further longitudinal studies are necessary to define the relative prognostic roles of morphology, nucleic acid cytometry, and surface phenotyping in NHL.

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