Nucleic Acid Flow Cytometry in Large Cell Lymphoma

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ABSTRACT

Between 1978 and 1985, 140 patients with large cell lymphoma (27 follicular, 92 diffuse, 5 immunoblastic, and 16 transformed) had DNA-RNA cytometry performed on involved tissue. DNA-RNA features were correlated with treatment outcome and compared to other established prognostic factors in 63 newly diagnosed patients who received uniformly intensive therapy. Significantly better outcome was noted for previously untreated patients with intermediate RNA content (RNA index, 1.0–1.8), diploid DNA content, and (during the initial 12-month follow-up) low proliferative activity. Of patients followed beyond 12–24 months, those with high proliferative activity appeared to have the most durable remissions, although this was not statistically significant. These findings suggested a preferential impact of intensive chemotherapy on patients with intermediate RNA content and possibly those with high proliferative activity, since previous studies and our own experience with relapsing patients have indicated a progressively worse outlook with higher proliferative activity and RNA index values. In newly diagnosed patients, multivariate analysis identified RNA content as the most important prognostic factor, followed by proliferative activity and serum lactate dehydrogenase. Thus, for patients with large cell lymphoma, DNA-RNA cytometry appears to be a valuable prognostic parameter for identifying a subset of patients who have a high likelihood of cure with intensive chemotherapy.

INTRODUCTION

Despite the clinical usefulness of histological classifications of the malignant lymphomas (1), patient outcome varies considerably in many of the subtypes, including LCL.3 The LCLs are particularly important because of their frequency and their potential curability. Numerous attempts have been made to stratify prognostic groups within the LCLs, including analysis of morphological subcategories (2–5), phenotypic subcategories (6, 7), clinical features such as stage and extent of disease (8–11), and cellular DNA content from paraffin-embedded tissue sections (12).

FCM of DNA content permits an objective and quantitative analysis of a large number of tumor cells with established correlations between DNA ploidy and chromosome number and between proliferative compartment size and labeling index (13–15). The derived information on DNA ploidy and proliferative activity has been shown to be a useful gauge of the aggressiveness of the malignancy for a number of solid tumors and hematological malignancies (16–21). AO provides a quantitative analysis not only of DNA but also of RNA content, both of which have been shown to relate to prognosis in the malignant lymphomas (22, 23). Thus, these studies add valuable biological data that can augment routine histopathological analysis.

In the present study, we extend the use of FCM of DNA content and provide the first extensive assessment of tumor cell RNA content in patients with LCL. The AO FCM studies reported here were done prospectively, using freshly biopsied specimens; the results were subsequently correlated with the patients’ clinical features and treatment outcome.

MATERIALS AND METHODS

Between 1978 and 1985, AO FCM analyses were performed on 140 biopsy specimens of LCL. Excluded from the remission analyses were three patients whose treatment response was uncertain. Excluded from remission and survival analyses were 10 patients who were known to have received either no therapy or suboptimal (e.g., single alkylating agent) therapy. Tissue was obtained by excisional biopsy in 98% of the patients and fine needle aspiration in 2%. The source of tissue was lymph node in 72% and involved extranodal solid tissue in 28%. Bone marrow, blood, and body fluid samples were not included in this analysis.

The biopsy material was reviewed at the time of the AO FCM analysis and classified according to the Working Formulation (1). Clinical features at the time of the AO FCM analysis were recorded and correlated with treatment outcome. In particular, distribution and bulk of nodal disease, extranodal involvement, and serum LDH level were assessed, in keeping with their known prognostic value for patients with newly diagnosed Stage III–IV DLCL (8).

The treatment approach was uniformly intensive for these patients, although specific therapy varied according to stage, current protocols, and, for relapsing patients, prior treatment history. All newly diagnosed patients received combination chemotherapy: 92% received doxorubicin-based programs; 43% also received VP-16/methotrexate noncross-resistant alternating combinations (24); 38% received chemotherapy in conjunction with radiotherapy (Stage I–III patients). Of relapsing patients, 43% received VP-16/methotrexate-based combinations (25), 19% received further doxorubicin-based combinations, 14% received cytara-bine-based combinations (26), 10% received Phase II single agents, and the remainder received a variety of other therapies.

The technique for AO FCM has been previously described (22, 27, 28). Specimens were minced and syringed to produce a single cell suspension. Representative involvement by lymphoma of the specimens was confirmed by examination of immediately adjacent tissue sections. An admixture of a fraction (not quantitated) of nonneoplastic lymphocytes in the specimens is expected; these background cells play a role in the morphological diagnosis of the lymphoma and likewise probably contribute to the ability of FCM studies to stratify the morphologic subtypes of lymphoma (22). Acridine orange staining was done according to a previously reported two-step procedure (27). Cytometric analysis was carried out with an ICP-22 mercury-arc cytometer (Phywe, Goettingen, W. Germany) with appropriate excitation and emission filters. At least 10,000 cells were measured in an optimum study. The median coefficient of variation of the G0–M DNA peak was 4.4% (range, 2.9–6.6). The analysis of the DI of the sample G0–M cells, the quantitation of cells in the S+G2M compartment, and the calculation of RI of the G0–M compartment were all done as previously described (22). Three technical points deserve comment: (a) The admixture of nonneoplastic cells can influence (usually reduce) the derived estimates of (S+G2M)% and, to a lesser extent, RNA index; (b) the (S+G2M)% estimate for
anaploid specimens derives from a more homogeneous neoplastic cell population and thus is less prone to this dilution effect than diploid populations; (c) the boxogram method of estimating \((S+G_2M)\)% is a satisfactory approximation for studies of freshly biopsied lymphoma samples in which the \((S+G_2M)\)% is generally low, but the same method would carry a high potential for error in situations of high proliferative activity, such as in some cell culture experiments. These issues have been addressed elsewhere (29–31). While these facts indicate potential limitations of the FCM-derived estimates of \((S+G_2M)\)% correlate well with tritiated thymidine labeling index (13-15).

RESULTS

Half of the FCM studies were done on specimens from newly diagnosed patients. Table 1 illustrates the FCM parameters according to histological category, for both newly diagnosed patients and those studied at time of relapse. The FLCL group according to histological category, for both newly diagnosed patients. Table 1 illustrates the FCM parameters

\[\text{Table 1 \ Nucleic acid features by histology}\]

<table>
<thead>
<tr>
<th>Histology</th>
<th>No. of patients</th>
<th>% Aneuploid</th>
<th>((S+G_2M)) % ± SD</th>
<th>RNA Index ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Previously Untreated</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Follicular LCL</td>
<td>18</td>
<td>61</td>
<td>8.1 ± 4.9</td>
<td>1.4 ± 0.8</td>
</tr>
<tr>
<td>Diffuse LCL</td>
<td>50</td>
<td>48</td>
<td>16.3 ± 8.6</td>
<td>1.8 ± 0.9</td>
</tr>
<tr>
<td>Immunoblastic</td>
<td>2</td>
<td>50</td>
<td>26.5 ± 3.5</td>
<td>1.8 ± 0.4</td>
</tr>
<tr>
<td>Relapsing</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Follicular LCL</td>
<td>9</td>
<td>56</td>
<td>5.9 ± 4.9</td>
<td>1.4 ± 0.5</td>
</tr>
<tr>
<td>Diffuse LCL</td>
<td>42</td>
<td>42</td>
<td>13.2 ± 8.8</td>
<td>1.8 ± 1.2</td>
</tr>
<tr>
<td>Immunoblastic</td>
<td>3</td>
<td>33</td>
<td>19.3 ± 16.2</td>
<td>3.1 ± 1.0</td>
</tr>
<tr>
<td>Transformed</td>
<td>16</td>
<td>50</td>
<td>11.5 ± 9.1</td>
<td>1.4 ± 0.7</td>
</tr>
</tbody>
</table>

and 44%. The FLCL group had a trend for longer survival \((P = 0.18)\) than the DLCL group. The two previously untreated patients with IBL both achieved CR and remain in remission with 9- and 12-month follow-up.

The overall CR rate for relapsing patients was 24%, and there were no significant differences among the histological categories. The median survival from relapse was 22 months for FLCL, 13 months for DLCL, 6 months for IBL, and 9 months for LCL transformed from low-grade lymphoma. The respective median FFSs were 7, 5, 3, and 4 months. For all histological categories, survival and FFS were shorter for patients with multiple relapses than for those in first relapse.

Newly Diagnosed Patients

DNA Index. Table 2 and Fig. 1 illustrate the analysis of the impact of DNA Index on attainment of CR, survival, and FFS for the 63 previously untreated patients who received intensive therapy. Patients with diploid LCLs had significantly better survival and FFS rates than patients with aneuploid malignancies.

RNA Index. Analysis of the study population in two cohorts, those above and those below the median RI value, failed to demonstrate prognostic differences. Since the lymphomas are diseases with a continuum of clinical behavior, and treatment failure is frequent both in the very indolent (low grade) and very aggressive (high grade) categories, similar heterogeneity was sought within LCL by dividing the population into three approximately equal groups. It then became apparent that patients with intermediate RNA content had the best prognosis (Table 2 and Fig. 2). Patients with very low RI had a slightly more indolent course than those with very high RI, but neither of these groups had a high proportion of durable remissions, whereas 80% of patients with RI 1.0–1.8 were projected to be failure free with a median follow-up period of 18 months. Both survival and FFS were superior for the group with intermediate RI, whether the analysis included all LCLs or was restricted to DLCL only.

Proliferative Activity. As with the analysis by RI, the analysis of survival and FFS by \((S+G_2M)\)% failed to demonstrate a single cutoff \((S+G_2M)\)% value that identified prognostically different groups (Table 3). Again, dividing the population into three groups revealed prognostically important differences.

Table 2 and Figs. 3 and 4 illustrate the analyses of the impact of \((S+G_2M)\)% on CR, survival, and FFS. For the overall group, low \((S+G_2M)\)% \((\leq10)\) was prognostically favorable, in part because of the large proportion of FLCL patients in the low \((S+G_2M)\)% group and their generally longer survival.

\[\text{Table 2 \ Outcome for previously untreated patients by nucleic acid features}\]

<table>
<thead>
<tr>
<th>Nucleic acid feature</th>
<th>DLCL Patients</th>
<th>All LCLs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% 2-Year CR</td>
<td>% 2-Year Survival</td>
</tr>
<tr>
<td>Ploidy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diploid aneuploid</td>
<td>21</td>
<td>79</td>
</tr>
<tr>
<td>RNA index</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤1</td>
<td>24</td>
<td>58</td>
</tr>
<tr>
<td>1–1.8</td>
<td>19</td>
<td>79</td>
</tr>
<tr>
<td>&gt;1.8</td>
<td>20</td>
<td>58</td>
</tr>
<tr>
<td>((S+G_2M))%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤10</td>
<td>13</td>
<td>77</td>
</tr>
<tr>
<td>10–18</td>
<td>14</td>
<td>50</td>
</tr>
<tr>
<td>&gt;18</td>
<td>18</td>
<td>75</td>
</tr>
</tbody>
</table>

\* See Fig. 2: No surviving patients with 24-month follow-up in this subgroup.
Fig. 1. Outcome by DNA index for patients with diffuse large cell lymphoma. Left, survival; right, failure-free survival.

Fig. 2. Outcome by RNA index for patients with diffuse large cell lymphoma. Left, survival; right, failure-free survival.

Table 3 Diffuse large cell lymphoma: survival by (S + G2M)% for previously untreated patients

<table>
<thead>
<tr>
<th>Cutoff (S + G2M)%</th>
<th>No. patients below/above cutoff</th>
<th>% 2 year survival below/above cutoff</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>13/32</td>
<td>38/43</td>
<td>0.19</td>
</tr>
<tr>
<td>12</td>
<td>17/28</td>
<td>35/44</td>
<td>0.53</td>
</tr>
<tr>
<td>14</td>
<td>21/24</td>
<td>29/50</td>
<td>0.88</td>
</tr>
<tr>
<td>16</td>
<td>23/22</td>
<td>33/49</td>
<td>0.68</td>
</tr>
<tr>
<td>18</td>
<td>27/18</td>
<td>27/60</td>
<td>0.59</td>
</tr>
<tr>
<td>20</td>
<td>29/16</td>
<td>24/68</td>
<td>0.25</td>
</tr>
<tr>
<td>22</td>
<td>34/11</td>
<td>32/64</td>
<td>0.69</td>
</tr>
<tr>
<td>24</td>
<td>35/10</td>
<td>33/60</td>
<td>0.91</td>
</tr>
</tbody>
</table>

When the analysis was restricted to DLCL patients only, patients with (S+G2M)% ≤10 were seen to have somewhat better short-term survival (particularly for the first 12 months) than other patients. However, the highest projected fraction of long-term survival and FFS was from the group with high (S+G2M)% (>18) (Fig. 4), although this was not statistically significant. The median follow-up of this high (S+G2M)% group with the highest apparent potential for cure was 15 months.

Multivariate Analysis. The nucleic acid features of the newly diagnosed patients (FLCL, DLCL, and IBL) of all stages were assessed along with the clinical features listed in the left half of Table 4. Prior analyses of Stage III–IV patients had shown the particular importance of serum LDH level and of tumor burden, which is a composite measure of number of extranodal sites and extent of nodal involvement (8). Because only three patients had T-cell LCL, the impact of phenotype could not be assessed reliably.

Table 4 Prognostic factors affecting survival in LCL using the Cox Regression Model

<table>
<thead>
<tr>
<th>Univariate analysis</th>
<th>P</th>
<th>Multivariate (in order of entry)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA Index (1.0–1.8 favorable)</td>
<td>0.001</td>
<td>RNA Index (1.0–1.8 favorable)</td>
<td>0.001</td>
</tr>
<tr>
<td>(S + G2M)% (10–18 adverse)</td>
<td>0.004</td>
<td>(S + G2M)% (10–18 adverse)</td>
<td>0.005</td>
</tr>
<tr>
<td>DNA Index (Diploid favorable)</td>
<td>0.007</td>
<td>LDH (≤225 mU/ml favorable)</td>
<td>0.001</td>
</tr>
<tr>
<td>Tumor burden (Low favorable)</td>
<td>0.02</td>
<td>Extranodal sites (Zero favorable)</td>
<td>0.03</td>
</tr>
<tr>
<td>Stage (I–II favorable)</td>
<td>0.03</td>
<td>LDH (≤225 mU/ml favorable)</td>
<td>0.06</td>
</tr>
<tr>
<td>Constitutional symptoms</td>
<td>NS*</td>
<td>Constitutional symptoms</td>
<td>NS*</td>
</tr>
<tr>
<td>Age</td>
<td>NS</td>
<td>Age</td>
<td>NS</td>
</tr>
</tbody>
</table>

which is a composite measure of number of extranodal sites and extent of nodal involvement (8). Because only three patients had T-cell LCL, the impact of phenotype could not be assessed reliably.

Table 4 summarizes the analysis of factors influencing survival. The univariate analysis identified six factors [RI, (S+G2M)%, DNA index, tumor burden, extranodal involve-
NUCLEIC ACID FLOW CYTOMETRY IN LARGE CELL LYMPHOMA

Fig. 3. Survival by (S+G2M)%. Left, all large cell lymphomas; right, diffuse large cell lymphoma.

Fig. 4. Failure-free survival by (S+G2M)% for patients with diffuse large cell lymphoma.

Relapsing patients

Table 5 summarizes the results for the 67 relapsing patients who received salvage therapy, according to their FCM features. The results for the subset with DLCL were similar. Low RI correlated significantly with longer survival and FFS. There was a trend for longer survival and FFS for those with low (S+G2M)%. DNA ploidy had no bearing on outcome for relapsing patients.

DISCUSSION

The current study demonstrates the prognostic utility of AO FCM in patients with large cell lymphoma. Each FCM parameter [RNA index, (S+G2M)%, and DNA index] provided significant prognostic information. This ability to stratify patients within one histological subtype of lymphoma indicates that AO FCM can be a valuable adjunct to routine histopathological examination of biopsy specimens. The dominant prognostic value of the RNA content in LCL has not previously been reported.

The RNA index was the strongest predictor of outcome in the current study. Cellular RNA content correlates with cellular protein content and cell cycle compartment; quiescent cells have the lowest amount of RNA (38, 39). In broad-based studies of malignant lymphoma, increasing RNA content has been noted to correlate with increasing histological grade and, consequently, with worse prognosis (22, 23). Likewise, in acute lymphoblastic leukemia, high RNA content correlates with an adverse prognosis (40-42). Our experience with relapsing LCL parallels these previous observations: within this one histological category, the natural history of the disease is most aggressive for high RNA-content LCLs.

In contrast, the pattern with previously untreated LCL showed a striking deviation from the expected worse prognosis with higher RI. In these patients, who received courses of intensive combination chemotherapy every 3 weeks, an intermediate RI was associated with the highest potential for cure, and patients with very high RI had virtually the same outcome as those with very low RI. Thus, LCLs with a high fraction of quiescent cells with low RI may be the least sensitive to this schedule of chemotherapy. Since low-grade lymphomas have a characteristically low RI (22, 23), it is possible that a low RI identifies a group of LCL patients who have a background of...
indolent lymphoma and who are thus less likely to be cured with currently available therapy.

In the present study, the presence of aneuploidy was an adverse prognostic factor for newly diagnosed patients. Aneuploidy has been associated with adverse outcome in a number of other malignancies (17), and in lymphoma aneuploidy is noted with increasing frequency as the histological grade increases (22, 23). Our findings contrast with the reports of Bauer et al. (12), who did not note any predictive role of DI, and Wooldridge et al. (43), who found patients had better response and survival rates with aneuploid LCLs than with diploid. The different findings in our series and Bauer's may be partly due to technical differences. In contrast to our use of AO on freshly biopsied tissue, they used PI or DAPI (4,6-diamidine-2-phenylindole dihydrochloride) to stain deparaffinized tissue. While generally reliable (44, 45), the resolution of Go1 DNA peaks of deparaffinized tissue is not as good as with fresh tissue. While AO gives slightly poorer resolution of DNA peaks than many DNA dyes, good concordance has been shown between AO and PI studies of fresh tissue (46), and the simultaneous quantitation of RNA provides not only the independently important RNA data, but also often permits separation on the basis of RNA content of different cell populations with similar or identical DNA contents. Both Bauer's and Wooldridge's series were retrospective while ours was prospective, but this difference does not readily explain the different results. While further studies of both archival material and freshly biopsied specimens will be needed to be certain of the impact of aneuploidy, our current data indicate that aneuploidy is an adverse prognostic factor in LCL.

The impact of (S+G2M)% on outcome in LCL appears to be twofold. First, there is a significant survival advantage for low (S+G2M)% LCLs, even when the analysis is restricted to DLCL. This finding is largely in accord with the findings of Bauer et al. (12) and Wooldridge et al. (43), although their emphasis was on the adverse outcome of patients with very high (S+G2M)% (>20), which included 42% of Wooldridge's patients, but only 15% of Bauer's. Our second and perhaps more provocative finding concerning (S+G2M)% was that patients with high (S+G2M)% (>18) may have the highest curative potential, based on the apparent 60% FFS plateau observed in these patients (Fig. 4). While this observation is based on relatively short follow-up and is not statistically significant, almost a third of the patients in the high (S+G2M)% group have been followed for over 24 months and are therefore past the time of highest risk for relapse. If continued follow-up confirms this impression, it will contradict the observation that highly proliferative lymphomas carry an adverse prognosis (47). Thus our data may indicate that chemotherapy has a particularly profound effect on the clinical course of highly proliferative LCLs.

Patients with relapsing LCL are generally incurable with current therapy. It is not surprising, then, that the prognostic implications of the FCM findings for relapsing patients were different than for newly diagnosed patients. No relapsing patients with a substantial chance of cure were identified. In the absence of potentially curative therapy, the FCM features that correlated with longer survival were those that correlate with a more indolent natural history: low RNA index and low (S+G2M)%.

The prognostic utility of AO FCM was compared with other known clinical features of prognostic significance through a detailed analysis of untreated patients. For LCL, a number of prognostically important clinical features have been well de-

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REFERENCES

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