ABSTRACT

Paraffin-embedded surgical biopsies from 50 patients with newly diagnosed diffuse large cell lymphoma (DLCL) were examined for proliferative activity and DNA aneuploidy by flow cytometry. These results were correlated with the clinical characteristics of these patients and the course of their disease. High proliferative activity, defined as less than 80% of cells in G0 or G1, was found to be the single most important pretreatment adverse prognostic factor in these patients. This relationship remained significant after correcting for poor performance status and advanced Ann Arbor stage, the other factors found to be associated with a shortened survival. DLCLs with high proliferative activity were more probable to present with extranodal involvement than those with lower proliferative activity. The mitotic count as determined by light microscopy did not correlate with flow cytometry-defined proliferative activity and may be a less accurate method for assessing this important biological characteristic in DLCL. DNA aneuploidy was detected in 62% of cases but did not appear to have any prognostic significance. Biopsies from patients who presented with lymphomatous bone marrow involvement, however, invariably demonstrated an aneuploid stemline. These results suggest that differences in proliferative activity may be an important biological basis for the variable prognosis seen in DLCL.

INTRODUCTION

Although DLCL is classified as intermediate grade in the International Working Formulation of non-Hodgkin's lymphomas, it shows considerable diversity in its clinical course. Complete remission and prolonged survival may be obtained with aggressive multiagent therapy, but approximately one-half of all patients with DLCL are unresponsive to chemotherapy or relapse after an initial remission. Various histological, immunological, and clinical characteristics have previously been reported to affect prognosis; nonetheless, the course of the disease in individual patients remains difficult to predict.

FCM can be used to rapidly assess cell proliferation and identify the presence of aneuploid stemlines within tumor specimens. This technique has been applied to single cell suspensions of non-Hodgkin's lymphoma by several investigators to study the biological diversity of these neoplasms. Other investigators have shown that the proliferative activity of non-Hodgkin's lymphomas as assessed by DNA content FCM correlates with the International Working Formulation grading of lymphomas. This relationship was recently confirmed by others who also found RNA content to be related to the International Working Formulation grade. In the present investigation, we attempt to confirm these findings of a biological basis for prognosis in non-Hodgkin's lymphomas through the comprehensive evaluation of 50 patients within one histological group.

We have studied the clinical and prognostic implications of proliferative activity and DNA aneuploidy in patients with DLCL. In contrast to previous studies of non-Hodgkin's lymphomas, we have analyzed specimens that were previously fixed and embedded in paraffin, using a technique similar to that originally described. This has enabled us to more rapidly evaluate the prognostic significance of our findings by studying cases in which the complete clinical course is known.

MATERIALS AND METHODS

Pathological Material. All pathological specimens analyzed in this study were previously obtained during the routine diagnostic evaluation of patients presenting to Northwestern Memorial Hospital. Tissues were fixed in buffered 10% formalin and processed using standard histological embedding techniques. Between January 1980 and May 1984, a total of 54 cases of DLCL were accrued, excluding specimens obtained at the time of relapse and patients in whom DLCL evolved from another lymphoid neoplasm. All cases were reviewed by our reference pathologist (D. V.) and classified according to the International Working Formulation (1); of note, cases of immunoblastic lymphoma were excluded from this study. The number of mitoses per 10 high power microscopic fields was determined in each case. Four μm sections of tissue adjacent to the material to be studied by FCM were stained with hematoxylin and eosin and examined to ensure the presence of lymphoma.

Seven paraffin blocks containing follicular hyperplasia were studied as controls; additionally, in two cases of DLCL and two cases of follicular hyperplasia, tissue was obtained from another lymphoid neoplasm. All cases were reviewed by our reference pathologist (D. V.) and classified according to the International Working Formulation (1). The unfixed cell suspension was thawed, stained simultaneously with the deparaffinized material, and evaluated in the same FCM run.

Tissue Deparaffinization, Rehydration, and Dissociation. Paraffin-embedded tissue was deparaffinized and dissociated using a previously reported method (15) of the method recently developed by Hedley et al. Briefly, three 30-μm sections of the paraffin block were cut and deparaffinized with xylene. The tissue was rehydrated in stepwise fashion with ethanol and washed three times with distilled water. The deparaffinized tissue was finely minced, suspended in 1 ml of 0.1% pepsin solution, and incubated for 30 min at 37°C. The pepsin proteolysis was stopped by the addition of 0.1 ml of peptatin A, and the digested material was filtered through 37-μm nylon monofilament mesh. HBSS-10 mM HEPES was added to the filtrate to a final volume of 15 ml. This mixture was centrifuged (all centrifugations are for 10 min at 200 x g, 4°C) and the nuclear pellet was resuspended in 2 ml of 10 mM HEPES-HBSS. Nuclear recovery was 3.16 × 10⁶ nuclei/g, with a range of 1.25 × 10⁷ to 7.04 × 10⁶ nuclei/g of deparaffinized tissue.
DNA Staining and Flow Cytometric Analysis. Following dissociation, the nuclei were stained using DAPI (Boehringer Mannheim Biochemicals, Indianapolis, IN) or PI (Calbiochem-Behring, San Diego, CA). For DAPI staining, dissociated nuclei were centrifuged and the nuclear pellets resuspended in DAPI (5 μg/ml) in 10 mM HEPES-HBSS, adjusted to a final concentration of 1–2 × 10^6 nuclei/ml, and incubated for 30 min at room temperature.

PI staining was performed as previously reported (16). The dissociated nuclei were resuspended in a solution of 0.1% Triton X-100 in phosphate buffered saline at a concentration of 1–2 × 10^6 nuclei/ml for 10 min at 4°C and then were centrifuged. The pellet was incubated with RNase A (180 units/ml; Worthington Biochemicals, Freehold, NJ) at a concentration of 1 × 10^6 nuclei/ml for 20 min at 37°C. The nuclei were then centrifuged and resuspended in an equivalent volume of a 50 μg/ml solution of PI in phosphate buffered saline. All DAPI- or PI-stained specimens were maintained in a light-shielded tube at 4°C for at least 1 h before FCM analysis.

The fluorescence of DAPI- and PI-stained cells was monitored on an EPICS V flow cytometer (Coulter Electronics, Inc., Hialeah, FL). Immediately prior to FCM analysis, the cell suspensions were filtered through a 37-μm nylon monofilament mesh filter. DAPI-stained cells were excited using an argon ion laser at 351–365 nm, 200 mW, and fluorescence was monitored through 457- to 485-nm interference and 470- to 480-nm long-pass filters. PI-stained cells were excited at 488 nm, 500 mW, and fluorescence was monitored through 515-nm interference and 515- to 530-nm long-pass filters. Fluorescent microspheres were used to assess instrument performance at the beginning of each run and as an external fluorescence standard. A minimum of 2.5 × 10^5 cells were evaluated from each specimen. The mean CV of the G0-G1 population in each sample for the entire study was 4.32, with a CV of 4.13 for those samples stained with DAPI and 6.27 for the four samples stained with PI. Analysis of the latter four specimens revealed higher quality DNA staining (i.e., smaller CV of G0-G1 peak) than observed following DAPI staining.

Comparison of biopsy material fixed in formalin or mercuric chloride/sodium acetate/formaldehyde demonstrated a consistently larger G0-G1 peak in the mercuric chloride-treated tissue; formalin-fixed tissue was therefore preferred for analysis. Prior to cell cycle analysis, a background of cellular debris which was visible in all histograms was eliminated by subtracting the area under an exponential curve from the DNA distribution (19). The "Simple Fit" program of Dean as adapted to the Terak 8600 (Terak Corporation, Scottsdale, AZ), with software developed by Salzman et al. (18) and extensively modified by Robinson and Leary of the University of Rochester. Prior to cell cycle analysis, a background of cellular debris which was visible in all histograms was eliminated by subtracting the area under an exponential curve from the DNA distribution (19). The "Simple Fit" program of Dean as adapted to the Terak 8600 (20) was used to estimate the distribution of cells within cell cycle compartments. This software uses a second-order polynomial to mathematically define the S-phase region of the cell cycle, with the remaining cells assigned to G0-G1 and G2-M regions. Cell proliferation in this investigation was expressed in terms of the percentage of G0-G1 cells. A protocol which derives the percentage of G0-G1 for the entire sample from the weighted average of the percentage of G0-G1 for each stemline was applied uniformly to those samples with mixed diploid and aneu-
PLOIDY AND CELL PROLIFERATION IN DIFFUSE LYMPHOMAS

Fig. 1. DNA content distributions of archival surgical pathology diffuse large cell lymphomas. A, specimen lacking a discernible aneuploid stemline and showing low proliferative activity; B, specimen lacking a discernible aneuploid stemline with high proliferative activity; C, specimen containing euploid and near-diploid aneuploid subpopulations.

Fig. 2. Frequency distribution of proliferative activity (percentage of Go-G1, cells) in diffuse large cell lymphomas. □, specimens obtained from patients not treated with curative intent and included in survival analysis. ■, specimens obtained from patients treated with curative intent and included in survival analysis.

Table 2 Response to chemotherapy

<table>
<thead>
<tr>
<th>CR*</th>
<th>No CR</th>
<th>P of differences in CR rates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Performance status</td>
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<td></td>
</tr>
<tr>
<td>Eastern Cooperative Oncology Group 0-1</td>
<td>17</td>
<td>5</td>
</tr>
<tr>
<td>Eastern Cooperative Oncology Group 2-4</td>
<td>0</td>
<td>4</td>
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<tr>
<td>Other extranodal disease</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>Absent</td>
<td>10</td>
<td>1</td>
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<tr>
<td>Proliferative activity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low (Go-G1 &gt; 80%)</td>
<td>15</td>
<td>6</td>
</tr>
<tr>
<td>High (Go-G1 ≤ 80%)</td>
<td>1</td>
<td>3</td>
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</table>

*CR, complete resolution of all clinically apparent disease.

analysis following the exclusion of specimens obtained from patients not treated with curative intent. There was no significant relationship between the presence of DNA aneuploidy, the DNA index, or the fraction of aneuploid cells in a sample and the proliferative activity.

There was no evidence of DNA aneuploidy in the nine follicular hyperplasia specimens obtained from paraffin blocks or previously frozen single cell suspensions. The DNA index was the same when single cell suspensions and nuclei from paraffin blocks were compared in the two cases (both aneuploid) of DLCL where both types of material were available. Comparison of the proliferative activity of the single cell suspension with their deparaffinized counterparts demonstrated a very close agreement in the percentage of Go-G1 in each case. A lower CV in the Go-G1 peak and less debris were consistently observed in the fresh/frozen specimen than in the deparaffinized samples (data not shown).

Clinical Characteristics. An Eastern Cooperative Oncology Group performance status of 0 or 1, i.e., fully ambulatory, and the absence of extranodal (other than bone marrow) involvement were predictive of a complete remission in patients treated with curative intent using combination chemotherapy (P = 0.008 and 0.034, respectively; see Table 2). Ann Arbor clinical stage, age, other clinical characteristics (the presence or absence of “B” symptoms, bulky disease, or marrow involvement), or histological findings (cleaved versus noncleaved nuclei, or sclerosis) did not significantly alter the chance of obtaining complete remission.

Among clinical parameters, survival was most strongly predicted by the performance status, with a median survival of 39 mo for patients who were fully ambulatory and only 11 mo for those at least partially bedridden (P = 0.003). Those with limited disease (Ann Arbor Stage 1 or II) also had a median survival of 39 mo, compared to a median survival of 14 mo for those with Stage III or IV disease (P = 0.046). The 29 patients whose tumors had cleaved nuclei had a slightly longer median survival than those with noncleaved nuclei (45 versus 39 mo; P = 0.056). The patients who presented with B symptoms had a median survival of 16 versus 39 mo for those who did not, but this difference failed to reach statistical significance (P = 0.097). There was not a statistically significant relationship between survival and age, marrow, or other extranodal involvement, bulky disease, or the presence of sclerosis in the biopsy.

Correlation between Proliferative Activity and Clinical Characteristics. The proliferative activity of the lymphoma as determined by DNA content FCM was the single most important pretreatment predictor of survival in this population. Lymphomas with high proliferative activity had a median survival of 7

performed in the first three cases described above and in two additional cases. In one instance, a small near-tetraploid population could not be separated from the diploid G2-M cells; and in the other, the contribution of background debris to the DNA histogram could not be estimated.

Fig. 2 illustrates FCM cell proliferation data for the DLCL cases. The mean percentage of cells in G0-G1 phase was 85.9% with a range of 51.5–98.3%. Forty cases had low proliferative activity (Go-G1 > 80%) and nine had high proliferative activity; 33 and 6 cases, respectively, were included in the survival...
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The proliferative activity remained a significant correlate of survival after correcting for performance status or Ann Arbor stage using Cox multivariate analysis or when only older patients were analyzed. After correcting for proliferative activity, however, performance status no longer significantly influenced survival, and the correlation of survival with Ann Arbor stage had only borderline significance ($P = 0.099$). Proliferative activity, in terms of the percentage of $G_0$-$G_1$ cells, also correlated significantly with survival when examined as a continuous function (i.e., without subdividing patients into two discrete groups of low and high proliferative activity), $P = 0.015$.

Low proliferative activity tended to increase the chance for complete remission in patients treated with aggressive chemotherapy (see Table 2), but this relationship was not statistically significant ($P = 0.12$).

High proliferative activity was related to extranodal (other than bone marrow) involvement by the lymphoma: 7 of 8 high proliferative activity tumors versus 18 of 37 low proliferative activity cases had such extranodal involvement ($P = 0.059$). The degree of proliferative activity in a specimen was not related, however, to whether the specimen itself was nodal or extranodal in origin. Large noncleaved cell lymphomas more frequently had high proliferative activity than did large cleaved cell lymphomas, but this relationship did not achieve significance ($P = 0.18$). Proliferative activity was not related to any other feature examined, including age, stage, performance status, B symptoms, or the presence of sclerosis in the specimen. Surprisingly, no relationship between the mitotic count determined by light microscopy and the proliferative activity as defined by DNA content FCM could be demonstrated.

Correlation between DNA Aneuploidy and Clinical Characteristics. Bone marrow involvement by the lymphoma was the only clinical characteristic significantly related to the presence of DNA aneuploidy. In all nine cases where marrow infiltration was demonstrated, the primary lymphoma specimen contained an aneuploid stemline ($P = 0.007$; see Table 3). Histological features (cleaved versus noncleaved nuclei, presence of sclerosis), age, stage, performance status, or the presence of B symptoms, bulky disease, or extranodal (other than bone marrow) disease were not significantly related to the presence of DNA aneuploidy; furthermore, overall survival and the complete remission rate was not influenced significantly by DNA aneuploidy, as shown in Fig. 4.

DISCUSSION

In this study, paraffin-embedded tissue was used to examine the relative prognostic significance of clinical, histological, and DNA content variables in patients with DLCL. Proliferative activity was found to be the single most important pretreatment determinant of survival (see Fig. 3). This relationship persisted even after correcting for other known prognostic factors such as age, stage, or performance status. The fraction of cells with $G_0$-$G_1$ DNA content also correlated well with survival when examined as a continuous function ($P = 0.015$). A previous study of 20 patients suggested a similar relationship between proliferative activity and survival in transformed noncleaved B-cell lymphomas, but this relationship was not statistically significant (9). Other investigators have recently reported that high proliferative activity had an independent adverse effect on survival in a group of 136 patients with various intermediate and high grade lymphomas (13).

In the past, proliferative activity in solid tumors has been estimated primarily by observing the frequency of mitotic figures in the histological section. We found no correlation between the mitotic count and proliferative activity as assessed by DNA content FCM. The mitotic count did not have any prognostic significance in a previous investigation of DLCL (24). Our finding of a striking relationship between FCM-defined proliferative activity and survival suggests that, at least for DLCL, FCM may provide a more useful estimate of biological aggressiveness than light microscopy. The aggressive nature of high proliferative activity lymphomas was also manifested by their tendency to present with extranodal involvement.

Analysis of DNA histograms of paraffin-embedded tissue requires a correction of cellular debris which otherwise would cause overestimation of the percentage of cells in $S$ phase (19).
as well as a method to estimate the total cell cycle distribution in mixed diploid-aneuploid populations. Currently available probes unfortunately do not allow for the separation of aneuploid and diploid populations on the basis of nuclear phenotypic characteristics associated with the aneuploid DNA stemline.

The mean percentage of cells in G0-G1 in the present study was 85.9%, which is in close agreement with previous reports of 84.8% G0-G1 cells in 51 cases of DLCL (13), 13% S-phase cells among large cleaved and immunoblastic lymphomas (25), and 13.2% S-phase cells in DLCL (12); furthermore, we found very similar proliferative activity when single cell suspensions and paraffin-embedded tissues from the same biopsy were compared. These results indicate that the DNA analysis protocol used in these studies permits accurate determination of cellular proliferative activity in paraffin-embedded DLCL specimens.

DNA aneuploidy was not found to affect survival in DLCL in this investigation (see Fig. 4). The presence of DNA aneuploidy has been reported to worsen the chance of attaining complete remission in previously treated patients (13). Biopsies obtained at relapse were excluded from our study because of the worsened prognosis associated with these cases. We did not find DNA aneuploidy to affect the chance of achieving an initial complete remission. Our results in DLCL contrast with previous investigations of epithelial cancers, where DNA aneuploidy has been associated with a worsened survival (26—28) and emphasize that the prognostic implications of aneuploidy in cancer cannot be generalized.

DNA aneuploidy appears to be a necessary condition for the development of bone marrow infiltration by DLCL, as seen in Table 3. A quantitative change of at least 2—3 chromosomes is generally required for the detection of DNA aneuploidy using current FCM instrumentation (29). This suggests that major abnormalities in the malignant karyotype may be required to adapt these lymphoid cells to the marrow microenvironment.

Using a conservative definition of aneuploidy, i.e., the presence of 2 distinct G0-G1 populations, we observed DNA aneuploidy in 62% of the 50 DLCL specimens. This is quite similar to the 61% frequency of DNA aneuploidy reported in 51 cases of DLCL (13) and the range of 50—86% reported by others in smaller numbers of large cell lymphoma specimens (9—12, 25).

The clinical characteristics found to be important prognostic factors in these patients with DLCL reflect the experience of others who have reported advanced stage and B symptoms to adversely affect survival (2, 3, 30, 31); similarly, the present investigation suggests improved survival in large cleaved cell relative to large noncleaved cell lymphomas, which is in agreement with some (4, 32), but not other previous reports (3, 5, 31, 33, 34).

In conclusion, proliferative activity as defined by DNA content FCM appears to be an important pretreatment predictive indicator of survival in DLCL. This observation must now be confirmed in larger studies using nuclei obtained from archival specimens where proliferative activity can be immediately correlated with prognosis and from single cell suspensions obtained at the time of diagnosis. Studies in which physical characteristics (7), RNA content (13), surface antigens (8), and nuclear antigens (15) are quantified along with DNA content have and will continue to provide valuable insight into the biology of lymphomas.

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Prognostic Implications of Ploidy and Proliferative Activity in Diffuse Large Cell Lymphomas

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