Prognostic Importance of Blast Cell DNA Content in Childhood Acute Lymphoblastic Leukemia

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Using flow cytometric techniques, we determined the pretreatment distribution of DNA content in propidium iodide-stained leukemic blasts from 205 children with "standard-risk" acute lymphoblastic leukemia (ALL). Risk assignment was based on an initial WBC count <100 x 10^9/L, no thymic mass, no meningeal leukemia, and leukoblasts lacking sheep erythrocyte receptors or surface immunoglobulin. A single aneuploid leukemic line was detected in 74 cases (36.1%): 70 hyperdiploid and four hypodiploid. For hyperdiploid cases, the DNA index (DI, or ratio of the DNA content of leukemic v normal G_0/G_1 cells) ranged from 1.06 to 2.0 (median, 1.20). A secondary leukemic line with hyperdiploid cellular DNA content was identified in 21 cases with diploid primary lines. Children whose primary leukemic line showed a DI \( \geq 1.16 \) (n = 57) had significantly better responses to treatment than did those with either a diploid DI (n = 130; P = .002) or values in the range of 1.01 to 1.15 (n = 14; P = .001). The relative risk of failure for hyperdiploid cases with DI \( \geq 1.16 \), corresponding to \( \geq 53 \) chromosomes, was one-third that of the other two groups. Treatment responses of patients with both diploid and hyperdiploid lines were identical to those associated with single diploid lines, but significantly worse than those associated with single hyperdiploid lines with DI \( \geq 1.16 \) (P = .016). The most favorable prognostic variables selected by a Cox proportional hazards model were: DI \( \geq 1.16 \) (P = .001), white race (P = .022), WBC \( \leq 25 \times 10^9/L \) (P = .032), age between 2 and 9 years (P = .075), and hemoglobin <7.0 g/dL (P = .094). DNA index \( \geq 1.16 \) retained its significant prognostic impact even after adjustment for other variables (P = .001). With the combination of DI \( \geq 1.16 \) and WBC \( \leq 25 \times 10^9/L \), one can identify a group of children with ALL who have a low probability of relapse when treated with current therapy. If they remain disease-free after longer follow-up, it may be advisable to treat them with less intensive, hence less toxic, chemotherapy than patients with higher WBC counts or lower DI values.

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ACUTE LYMPHOBLASTIC leukemia (ALL) is thought to result from the clonal expansion of lymphoid progenitors transformed at various stages of differentiation.1-3 Certain features of leukemic cells, such as the differentiation phenotype and specific immunoglobulin gene rearrangements,4 apparently reflect normal lymphoid cell properties that are retained in the malignant state. Other features, such as karyotypic abnormalities5 and activated cellular protooncogenes,6 are linked to the process of malignant transformation, but in ways that remain poorly understood. A logical extension of these studies has been the attempt to identify the biologic features of leukemic cells that are most closely related to prognosis. In childhood ALL, such diverse features as immunophenotype,7,8 percentage of S-phase cells,9,10 and ploidy as determined by karyotypic analysis11,12 have all been shown to convey important prognostic information. In one recent study, ploidy (or chromosome number) was the strongest single predictor of outcome and the only variable that added significant prognostic information to leukocyte count.11

Flow cytometric (FCM) analysis of cellular DNA content is a powerful tool for assessing prognostic variables in lymphoproliferative malignancies in adults.13-16 With this technique, the DNA ploidy of malignant G_0/G_1 cells and the percentage of S-phase cells can be determined. In adults with lymphoma,13,14 mycosis fungoides,15 and myeloma,16 both aneuploidy and a high percentage of S-phase cells determined by FCM analysis have been associated with unfavorable responses to therapy. In an FCM study of blast cell DNA content distribution among 173 children with ALL, we found that both the frequency of aneuploidy and the percentage of S-phase cells varied with the biologic subtypes of this disease.17 We now report the prognostic importance of these variables.

MATERIALS AND METHODS

Patients and Treatment

From Aug 2, 1979, through May 9, 1983, 261 children with newly diagnosed ALL had clinical and laboratory features that qualified them for therapy according to our Total Therapy Study X—Standard Risk protocol.18 Specifically, they had WBC counts <100 x 10^9/L, no mediastinal mass, lymphoblasts lacking sheep erythrocyte receptors or surface immunoglobulin, and showed no evidence of initial CNS disease. Patients with these features accounted for 73% of those with ALL admitted during the study period.

Marrow blasts were collected before treatment from each child, and DNA content studies were successful in 205 (79%) of the cases.
classified as standard-risk ALL. The percentage of S-phase cells could be assessed in 191 patients with ≥60% replacement of marrow by leukemic blasts. Sufficient leukemic cells were available for surface phenotyping in 191 patients, and cytogenetic studies were adequate to determine the ploidy of the leukemic stem line(s) in 147 patients. These investigations were part of a larger leukemia cell profile study approved by the institution's Clinical Trials Committee.

Therapy specified by the Total Therapy Study X—Standard Risk protocol consisted of conventional three-drug remission induction (vincristine, prednisone, and asparaginase) followed by either oral 6-mercaptopurine and methotrexate maintenance therapy with intermittent high-dose intravenous (IV) infusions of methotrexate or three alternating drug pairs for 18 months. Both regimens concluded with 12 additional months of oral 6-mercaptopurine and methotrexate. Follow-up observations have so far indicated no important differences in outcome for standard-risk patients treated in either arm of this study.18

**DNA Content by FCM**

Leukemic marrow samples were stained with a DNA-specific dye, propidium iodide, and analyzed by flow cytometry as previously described.15 DNA content histograms were analyzed for DNA index (DI, ratio of the channel numbers of leukemic and normal G0/G1 cells), percentage of S-phase cells, and 4N index (ratio of 4N and mid S-phase cells) as described.17 A leukemic line with a DI of 1.0 was determined to be present if the percentage of diploid G0/G1 cells was ≥20% more than the percentage of non-blast cells by differential count of Wright-stained cells. For cases with multiple lines, the leukemic line with lowest ploidy was arbitrarily designated as the primary line. A second tetraploid line in the G2+M-phase region of the primary line was detected for histograms with >2.0% of S-phase cells if the 4N index was >2.6 for the TPS-1 or >4.1 for the EPICS-V flow cytometer, as previously described.17

**Cell Surface Phenotype**

Lymphoblast surface phenotype was determined from reactivity with specific heteroantisera or monoclonal antibodies and formation of rosettes with sheep erythrocytes. The samples were classified as common, T cell, or undifferentiated, based on the pattern of lymphoblast surface markers.17

**Cytogenetic Studies**

Bone marrow samples were analyzed by use of a direct technique developed for acute lymphoblastic leukemia and a modified trypsin-Giemsa technique for chromosome banding.19

**Statistical Analysis**

Times to failure, as of Feb 1, 1984, were analyzed by the Kaplan-Meier method20 and the resulting curves compared by the log-rank test.21 Time to failure was defined as the interval between achievement of remission and relapse or death from any cause. Patients who did not fail were censored at the time of last follow-up. Patients who failed to enter remission were counted as failures and were assigned a failure time of zero.

The influence of potential prognostic factors on remission duration was estimated with the proportional hazards model of Cox,22 for those patients who attained a complete remission. Variables to be analyzed were dichotomized and recoded as 0 ("better" category) or 1 ("worse" category). Each factor was then tested as a single regressor variable in the Cox model, using the likelihood ratio test. The relative magnitude of a factor's effect on remission duration was estimated by computing the ratio of hazard function for the "worse" v "better" categories. Any factor yielding a P value of <.10 was considered for inclusion in the multivariate Cox model. Final selection depended on whether or not the P value was <.10 after adjustment for other variables in the model. A significance level of .10 was chosen to lessen the chance of excluding features that might influence the role of DI.

The relationships of various clinical features to DI categories, percentage of S-phase cells above or below the median, and induction response were tested by use of Pearson's chi-square or Fisher's exact test (two-sided). The differences in median percentage of S-phase cells among DI groups were tested with the Kruskal-Wallis test and associated multiple comparisons procedures.

**RESULTS**

**DNA Index and Prognosis**

The distribution of the primary leukemic line DI values in this series is shown in Fig 1. Most cases had G0/G1 DNA content, which by FCM analysis was indistinguishable from that of normal diploid cells (DI = 1.0). Hypodiploid DNA content was detected in only four cases. Hyperdiploid DNA content was evident in 34.6% of cases and showed a unimodal distribution (median DI, 1.20). Two patients had single leukemic stem lines with near-tetraploid DNA content.

Multiple leukemic lines were detected in 25 (12%) of cases, as previously reported.17 Three of the four hypodiploid cases had secondary leukemic lines with twice the DNA content of the primary line. Twenty-one of the remaining cases had diploid primary lines with hyperdiploid secondary lines, often in the tetraploid range. A single case had a DI of 1.07 for the primary line and a DI of 2.10 for the secondary line.

Patients were grouped according to the leukemic line of lowest ploidy for determination of the influence of DI on prognosis. Hyperdiploid proved to be a favorable prognostic feature, and a DI cut point of 1.16 yielded the best discrimination of the group with a low failure rate (Fig 2). The distribution of patients by DI

![Fig 1. Pretreatment distribution of DNA index (DI) for the primary leukemic lines of 205 patients with ALL.](image-url)
DNA CONTENT IN CHILDHOOD ALL

Fig 2. Time-to-failure curves for 201 patients with DNA index (DI) equal to 1.0 (diploid); ranging from 1.01 to 1.15; or ≥1.16. The patients with DI ≥ 1.16 had significantly better responses than the patients with diploid (P = .002) or intermediate (P = .001) DI. Responses were similar for patients with diploid and intermediate values (P = .316).

was similar for the two treatment groups on the Total Therapy Study X—Standard Risk protocol (P = .152). The presence of a diploid leukemic line indicated a high failure rate, even in the presence of additional leukemic lines with hyperdiploid DNA content (Fig 3). There were too few patients with hypodiploid cellular DNA content for meaningful analysis. Of the four patients in this category, one failed to achieve remission and two have failed after nine and 37 months of therapy, consistent with our impression that such cases have an unfavorable prognosis.

Fig 3. Time-to-failure curves for 185 patients with single diploid lines; multiple lines, including diploid and hyperdiploid (DI ≥ 1.16) lines; and single hyperdiploid (DI ≥ 1.16) lines. The patients with multiple lines had significantly higher failure rates than did those with single hyperdiploid lines (P = .016), and similar failure rates compared with patients having single diploid lines (P = .966). Excluded from the analysis were 14 patients with primary line DNA index (DI) of 1.01 to 1.15 and 2 patients with diploid primary lines and second lines with DIs of 1.01 to 1.15.

DNA Index and Ploidy

As previously noted,17 there was good agreement between the DI and the ploidy classification determined by cytogenetic analysis. Chromosome numbers and corresponding DI values of the lowest ploidy leukemic stem lines for 147 cases with successful cytogenetic studies are shown in Table 1. The group with DI ≥ 1.16 corresponded generally to the group with ≥53 chromosomes. Some exceptions were noted but were not unexpected, as individual human chromosomes vary up to 4.5-fold in DNA content.24 Two cases were found to have hyperdiploid stem lines despite karyotypes with only normal metaphases, probably because of the low mitotic index of leukemic cells in these cases compared with residual normal marrow cells.17

Percentage of S-Phase Cells

For marrow samples that showed ≥60% replacement by leukemic blasts, we estimated the proliferative fraction in terms of percentage of cells in S-phase. The median percentage of S-phase cells was 6.8, with a broad distribution skewed toward higher values (Fig 4). As previously reported,17 the median percentage of S-phase cells was higher for patients with single hyperdiploid rather than single diploid stem lines (9.5% v 6.6%; P < .05). An unexpected finding was that patients with multiple stem lines had significantly lower percentages of S-phase cells (median, 4.6%) than did all other groups (P < .05).

Table 1. Comparison of DNA Index (DI) With Karyotype Ploidy

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*For cases with multiple lines, the lowest ploidy line detected by each technique is shown.
†This patient had both a pseudodiploid and a 54 chromosome line detected by karyotyping.
‡Both patients were studied with the TPS-1 flow cytometer, which had lower resolution than the EPICS V flow cytometer.17
Fig 4. Distribution of the percentage of S-phase cells at diagnosis for 191 patients with ≥60% marrow replacement by lymphoblasts.

The probability of treatment failure was found to be significantly greater for patients with percentages of S-phase cells below the median value ($P = .016$, Fig 5). Further analysis revealed that the increased failure rate could be explained by the eight induction failures, all of whom had values below the median. The probability of failure after complete remission was similar for patients with percentages of S-phase cells above or below the median value. Percentages of S-phase cells for patients who did not respond to induction therapy (range, 0.6% to 5.9%) did not differ from values for other patients in the ≥6.8 group.

Clinical Features

Table 2 shows the clinical features of patients according to DI and percentage of S-phase cells. Patients with single hyperdiploid stem lines (DI ≥ 1.16) were predominantly in the 2 to 9 years age group. In addition, all but two of the hyperdiploid patients tested were found to express the common ALL antigen (CALLA). When divided according to median percentage of S-phase cells, the group with values equal to or less than the median contained a higher proportion of patients 10 years or older with WBC ≥ 25 × 10^9/L ($P < .05$).

Multivariate Analysis

In view of the prognostic importance shown for DI and percentage of S-phase cells as single variables, it was important to evaluate these factors in the context of other known prognostic features in ALL. When the DI categories, percentage of S-phase cells, and clinical features shown in Table 2 were analyzed for their ability to predict failure of response to induction therapy, only percentage of S-phase cells ≤6.8 ($P = .007$) and age <2 or ≥10 ($P = .022$) achieved significance. Unfortunately, however, the eight patients who failed induction therapy could not be distinguished from the other 88 patients with percentage of S-phase cells ≤6.8 who achieved remission.

The influence of clinical and laboratory features on the duration of complete remission after a favorable response to induction therapy was analyzed with the Cox proportional hazards model (Table 3). DI ≥ 1.16, race, WBC, hemoglobin, and age each achieved significance as single variables and each contributed independent prognostic information in the multivariate model ($P < .10$). The presence of detectable CALLA on the blast cell surface was a favorable prognostic feature when analyzed as a single variable ($P = .069$), but was no longer significant after adjustment for other prognostic variables ($P = .41$). In this patient population, with WBC < 100 × 10^9/L, DI ≥ 1.16 was the most significant prognostic variable in the model ($P = .001$). Even after adjustment for the influence of other variables, patients with DI ≥ 1.16 had a relapse hazard rate one-third that of those with lower values.

The gain in prognostic information obtained by combining DI with WBC can be readily appreciated from the Kaplan-Meier analysis depicted in Fig 6. Of the 144 patients with WBC ≤ 25 × 10^9/L, the group
Table 2. Distribution of Clinical Features According to DNA Index (DI) and Percentage of S-Phase Cells

<table>
<thead>
<tr>
<th>Clinical Feature</th>
<th>No. of Patients by DI*</th>
<th>No. of Patients by Percentage of S-Phase Cells</th>
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<tr>
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<td>1.01-1.15 (Single)</td>
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<tr>
<td>Sex</td>
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<td>WBC (x 10^6/L)</td>
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<td>Hb (g/dL)</td>
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<td>Lymphoid phenotype</td>
<td>93</td>
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*Divided to separate those patients with single diploid lines from those with a diploid lowest ploidy line and a second line with a DI ≥ 1.16. Only three patients could not be classified.

with DI ≥ 1.16 had a 4.2-fold lower hazard rate of failure than those with DI = 1 to 1.15 (P = .001). The prognostic importance of DI ≥ 1.16 was confined to the group with low WBC; there was no difference in failure rates for patients with WBC ≥ 25 x 10^6/L when they were divided into groups based on DI.

**DISCUSSION**

In this report, we demonstrate that FCM analysis of cellular DNA content can be used to identify a subset of childhood ALL patients with hyperdiploid leukemic lines and a favorable prognosis. Because of the large number of patients studied, we were able to refine the

Table 3. Relationship of Selected Variables to Duration of Complete Remission

<table>
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<tr>
<th>Variable</th>
<th>A (Better)</th>
<th>B (Worse)</th>
<th>P Value From Likelihood Ratio Test*</th>
<th>Estimated Ratio of Hazard Function†</th>
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<td>Other (n = 50)</td>
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<td>≤6.8 (n = 88)</td>
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<td>CALLA- (n = 17)</td>
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<td>Multivariate model</td>
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*Determines whether, for a given variable, the proportional hazards model provides a better fit with remission duration than does a model without the variable. For multivariate analysis, the test was applied to a model that included DNA index (DI), race, WBC, Hb, and age as covariates compared with one lacking the indicated variable.

†The ratio of hazard function gives the estimated proportional increase in risk of relapse at any given time for a patient having the "worse" category of a feature relative to one having the "better" category. For example, a patient with a DI < 1.16 is three times more likely to relapse than a patient with a DI ≥ 1.16. In the multivariate model, these values represent the relative hazard ratios for patients differing only in categories of the indicated variable.

‡Percentage of S-phase cells and phenotype could not be determined for some patients.
measurements are not affected by the proliferative rate and 44 to 48 chromosomes cannot be reliably distinguished techniques and instrumentation, however, cases with mitotic index of leukemic cells. '3' Clearly, even with newer 1084

Fig 6. Time-to-failure curves for 201 patients divided into groups based on primary leukemic line DNA index (DI) values of ≥1.16 or 1.0 to 1.15. In panel A, the results are shown for patients with WBC ≤ 25 × 10⁹/L. In this group, patients with DI ≥ 1.16 had a 4.2-fold lower hazard rate of failure than did patients with DI 1.0 to 1.15 (P = .001). In panel B, the results are shown for patients with WBC > 25 × 10⁹/L; in these patients there was no difference in failure rate for the subgroups based on DI (P = .38).

definition of the "favorable group" to those with DI ≥ 1.16, corresponding to ≥53 chromosomes in the leukemia cell karyotype. In cases with multiple leukemic lines, the prognostic importance of DI was determined by the line conferring the worst prognosis (Fig 3). In practice, the correct prognostic designation can be made by classifying each case according to the leukemic line of lowest ploidy, which we have arbitrarily termed the "primary" line.17

Flow cytometry has definite advantages over karyotyping for the detection of hyperdiploid cases: it is much more rapid and automated, an interpretable reading can be obtained for most patients, and measurements are not affected by the proliferative rate and mitotic index of leukemic cells.13,17 Even with newer techniques and instrumentation, however, cases with 44 to 48 chromosomes cannot be reliably distinguished from diploid. This poses no serious problem in risk-
group assignment, since the favorable group is the one with ≥53 chromosomes. The major disadvantage of FCM analysis is that it does not detect pseudodiploid cells with balanced chromosomal translocations, which do not affect the net DNA content of cells. Translocations are an important unfavorable prognostic feature in childhood ALL,4,11,25,26 but have not been observed in ALL cases with >50 chromosomes,4 indicating that the DI ≥ 1.16 group does not overlap with the translocation group. Thus, karyotyping and FCM analysis of cellular DNA content are complementary techniques in the evaluation of patients with childhood ALL. In our view, leukemic cells from each newly diagnosed patient should be analyzed by both methods.

Childhood ALL was the first malignant disease in which hyperdiploidy was found to be a favorable prognostic variable.11,12 Recently, we showed that hyperdiploid cellular DNA content predicts a favorable response to cyclophosphamide and doxorubicin in infants with unresectable neuroblastoma.27 By contrast, the prognosis for adults with lymphoproliferative malignancies or solid tumors has generally been worse for cases with hyperdiploid DNA indices.13 Clearly, the relative prognostic importance of the ploidy of malignant clones must be assessed independently for each human tumor type, as a function of age, in the context of prospective treatment protocols.

The overall remission induction rate in this study was 94%, as would be expected for children with "standard-risk" ALL treated with modern chemotherapy. Despite the low initial failure rate, we were able to demonstrate a correlation between low percentage of S-phase cells (below the median of 6.8%) and the lack of response to induction therapy. This finding is consistent with results reported in adult acute leukemia,28-30 and probably has not been recognized previously in "standard-risk" childhood ALL because of the large number of patients required to study induction failures. However, since the percentages of S-phase cells for patients who fail induction overlap with values less than the median for patients who do achieve remission, this determination cannot be used prospectively as a prognostic variable.

The finding of a low percentage of blast cells in S-phase among patients with "standard-risk" features who fail induction has interesting theoretical implications. Because most antineoplastic drugs are preferentially cytotoxic to proliferating cells, these patients may fail because of the persistence of indolent, slowly growing blasts, rather than overgrowth of drug-resistant mutants. Thus, a failure of initial response may have quite different implications for "standard-risk" ALL than for B cell ALL, for example, which has a high proliferative rate and exhibits rapid early response followed by rapid regrowth of resistant cells.
Our experience has been that a significant proportion of induction failures with standard-risk ALL have achieved remission after additional VM-26 and ara-C therapy, and have had long remissions on standard maintenance schedules. Low blast cell proliferative rate as a cause of initial induction failure in “standard-risk” ALL is consistent with these results of extended therapy.

The impact of pretreatment percentage of S-phase cells on remission duration in acute leukemia has been controversial. In this study, we found no difference in relapse hazard for cases with percentage of S-phase cells above or below the median value, in contrast to Scarffe et al9 and Dow et al,10 who reported an independent, adverse prognostic significance of percentage of S-phase cells (or labeling index) >6%. This lack of agreement is best explained by differences in therapy and patient selection.

The interrelationships among prognostic features in childhood ALL have emphasized the need for multivariate analysis to determine those variables with independent significance. In this study, DI, race, WBC, hemoglobin, and age had significance when analyzed as single variables, and each contributed independently in the multivariate model (Table 3). The presence or absence of CALLA as determined by qualitative immunofluorescence analysis, was not an independent significant variable, confirming our previous results6 and those of Greaves et al.11 However, dual-parameter FCM measurements of CALLA expression by cells in S-phase indicate that increased amounts of antigen correlate independently with longer remission durations,32 although the biologic basis for this association remains unknown.

The projected overall results of this study indicate a need to intensify therapy, following the example of Riehm et al in the BFM trials.33 However, using flow cytometry to measure cellular DNA content in combination with WBC, we have identified a group of ALL patients who have a low relapse hazard when they are treated with current therapy. If they remain disease-free after longer follow-up, it may be advisable to treat them with less intensive, hence less toxic, chemotherapy than patients with higher WBC or lower DI values.

ACKNOWLEDGMENT

We thank Dr Sue Melvin for performing the surface phenotype analysis, and Mr John Gilbert for editorial review and critical comments. We acknowledge the expert technical assistance of Edward Wingfield, L. Keith Brown, Graydon Nelson, Barry Somerville, Ann Tipler, Elsie White, Kenna Williams, Ann Harris, Terri Flake, Patricia Ball, and Elizabeth Mann.

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Prognostic importance of blast cell DNA content in childhood acute lymphoblastic leukemia

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