Aneuploidy and Percentage of S-Phase Cells Determined by Flow Cytometry Correlate With Cell Phenotype in Childhood Acute Leukemia

By A. Thomas Look, Susan L. Melvin, Dorothy L. Williams, Garrett M. Brodeur, Gary V. Dahl, David K. Kalwinsky, Sharon B. Murphy, and Alvin M. Mauer

Acute leukemia is subdivided into lymphoblastic (ALL) and nonlymphoblastic (ANLL) categories, based on cellular morphology. Childhood ALL is a heterogeneous disease with variable expression of specific differentiation antigens on the malignant cell surface. Based on the state of differentiation arrest of the malignant clone, lymphoblasts have been divided into common (C), thymus-derived (T), surface-immunoglobulin-positive (B), and undifferentiated (U) categories. These subtypes have therapeutic as well as biologic significance, since patients with T- and B-cell ALL have a poor prognosis.

Clonal abnormalities of the DNA content of leukemia cells can be assessed by flow cytometry (FCM) after staining with specific fluorochromes. Aneuploidy detected by FCM is a specific marker of malignant disease with variable expression of DNA content distributions in childhood acute leukemia and could well provide insight into the heterogeneity of this disease. Therefore, we studied the DNA content distributions in childhood acute leukemia and determined that both the frequency of aneuploidy and the percentage of S-phase cells varied with the biologic subtypes of this disease.

MATERIALS AND METHODS

Patients

From August 2, 1979 to January 17, 1982, 280 patients with acute leukemia were admitted to St. Jude Children's Research Hospital. Based on cytology and specific staining characteristics of the blasts, 224 had ALL and 56 had ANLL. Cell morphology was classified by 3 independent reviewers according to the criteria of the French-American-British Cooperative Group (FAB). DNA content studies by FCM and surface phenotype classification were successful in 173 with ALL; DNA content studies were also successful in 52 patients with ANLL. In 193 of these cases, cytogenetic studies were adequate to determine the modal chromosome number of the leukemia stem line. These investigations were part of a cell profile protocol study approved by the Clinical Trials Committee of this center.

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DNA Content by FCM

To minimize blood contamination, a small amount (0.1–0.3 cc) of leukemic marrow was obtained from each patient into Na+-citrate solution and the fluorescence >590 nm emitted from the slides. These samples were stained in hypotonic propidium iodide for the case with a single diploid stem line, the 4N was <4.1, indicating only G0 + M cells in the second peak (B); the other 4N was > 4.1, indicating G0/G1 cells from a second tetraploid stem line in the G0 + M region of the tetraploid stem line (D). Tetraploid S-phase cells are evident, extending off-scale on the enlarged histogram (D).

DNA content histograms from marrow samples were analyzed for the DNA index (DI) and coefficient of variation (CV) of the G0/G1-phase modal channel numbers of the leukemic and diploid cells. Only those histograms with clearly defined diploid and leukemic G0/G1 populations in the marrow sample were considered to have an abnormal DI. The CV of the fluorescence distribution of G0/G1-phase leukemia cells was determined from the formula CV = (HM × 100) / (N × 2.35), where HM was the width of the G0/G1 peak at half-maximum height and N was the modal channel number of the G0/G1 peak. For cases with multiple stem lines, an arbitrary designation of primary, second, and third stem lines, respectively, indicated lowest to highest DNA content per cell.

To detect a second tetraploid leukemia stem line in the G0 + M-phase region of the primary stem line, we calculated a 4N index (4NI) for the 4N cells divided by mean height of mid-S-phase cells) from enlarged histograms of both samples distinguishes the one with a second stem line. For the case with a single diploid stem line, the 4N was <4.1, indicating only G0 + M cells in the second peak (B); the other 4N was > 4.1, indicating G0/G1 cells from a second tetraploid stem line in the G0 + M region of the pseudodiploid stem line (D). Tetraploid S-phase cells are evident, extending off-scale on the enlarged histogram (D).

EPICS-V flow cytometers. Histograms with 4NI >3.0 SD above these means (>2.7 for the TPS-1 and >4.1 for the EPICS-V) were considered to have G0/G1 cells from a second stem line overlapping the G0 + M region of the primary stem line. A leukemia stem 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 nonblast cells in the Wright-stained differential count.

The percentages of cells in the G0/G1, S, and G0 + M phases were determined from analysis by a computer program called PEAK, generously given to us by Dr. Phillip N. Dean.22,23 The percentage of S-phase cells was evaluated for 210 of the 225 cases with >60% blasts by differential count. When there were multiple stem lines, G0 + M cells of the 2N stem line could not be distinguished and were not subtracted from 4N G0/G1 cells. The same methods were used to evaluate the second stem lines of near-haploid (1N) primary stem lines. In cases with multiple stem lines, the percentage of cells in S-phase that was used for analysis was the sum of the percentages of S-phase cells in each stem line.

Cell Surface Phenotype

Lymphoblast surface phenotype was determined from reactivity with specific heteroantisera and formation of rosettes with sheep erythrocytes (E). The C-ALL, Ia-like, and T antigens were detected

<table>
<thead>
<tr>
<th>Table 1. Identification of ALL Subtypes by Membrane Markers</th>
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<tr>
<td>Membrane Marker</td>
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<tr>
<td></td>
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<tr>
<td>Erythrocyte receptors</td>
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<tr>
<td>T-cell antigens</td>
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<tr>
<td>Common ALL antigen</td>
</tr>
<tr>
<td>Ia-like antigens</td>
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<tr>
<td>Surface immunoglobulin</td>
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</tbody>
</table>

C, common ALL; T/C thymus cell ALL, C-ALL antigen positive; T/E-, thymus cell ALL, erythrocyte receptor negative; T/E+, thymus cell ALL, erythrocyte receptor positive; B, surface-immunoglobulin-positive ALL; U, undifferentiated ALL; + present, - absent.
RESULTS

DNA Index and Ploidy

A typical DNA content histogram of marrow cells from a patient with hyperdiploid ALL is shown in Fig. 2. All patients with aneuploid leukemia had a small population of residual G0/G1-phase diploid cells, as shown in the figure. The diploid marrow cells sometimes caused a skew in the peak of G0/G1 leukemia cells that was resolved into a distinct second peak in the histogram for the mixture of leukemic plus normal blood cells.

Comparison of the leukemia cell DI with the karyotype showed that the FCM-detected abnormalities were primarily due to gains or losses of chromosomes of average size (Fig. 3). The sensitivity of detecting aneuploidy differed with the model of flow cytometer used. This was because the CV (mean ± SD) of the 106 samples studied with TPS-1 (5.6 ± 0.9) was greater than the CV of the 119 samples studied with the EPICS-V (2.7 ± 0.8) (p < 0.001). Due to this difference in sensitivity, stem lines with >48 chromosomes were consistently detected with the EPICS-V compared to stem lines with >52 chromosomes with the TPS-1.

Two cases had an FCM-detected abnormal DI, despite diploid karyotypes; one had B-cell leukemia with the characteristic 14q+ translocation and a DI of 1.07. The second case appeared to have a normal karyotype, although the leukemia stem line (DI = 1.2)
may have been missed, because only 12 metaphases could be scored.

DNA Index and Leukemia Cell Phenotype

Based on 225 patients classified by leukemia-cell phenotypes (Fig. 4), an abnormal DNA index was more frequent in ALL (30.1%) than in ANLL (3.8%) (p < 0.001). Within the ALL group, aneuploidy was most frequent in the C-ALL subtype. Of the 120 patients with C-ALL, 35.8% were aneuploid, and all but one were hyperdiploid; the median abnormal DI was 1.20, and 60% of the abnormal values were between 1.15 and 1.25. In the T/C-ALL subgroup, 3 of 7 cases were aneuploid; one had a near-haploid clone (DI = 0.59) and is detailed in a separate report.25 In the T-ALL group, aneuploidy was much less frequent (1 of 12 E− and 1 of 9 E+ patients), and the combined frequency was significantly less than it was in the C-ALL group (p = 0.02).

In the ALL group, the frequencies of aneuploidy...
were similar for the FAB morphological groups: 42 of 135 for L1 (0.31), 9 of 33 for L2 (0.27), and 1 of 5 for L3. In the ANLL group, the patient with haploid leukemia had promyelocytic (M3) morphology, and the patient with hyperdiploid leukemia had myelomonocytic (M4) morphology.

**S-Phase Cell Percentage and Leukemia Cell Phenotype**

The percentage of S-phase cells of leukemic marrows varied widely from patient to patient, even within groups with the same leukemia cell phenotype (Fig. 5). B-ALL had a significantly higher percentage of S-phase cells (29.8%) than did any other group (p < 0.03). The median percentage of S-phase cells of the T/E+ group (13.3%) was next highest, being significantly greater than the medians of the remaining phenotype groups (p < 0.03).

The C-ALL group had sufficient aneuploid patients to analyze the relationship between S-phase cell percentages and ploidy. The median percentage of S-phase cells was significantly greater for hyperdiploid (8.4, n = 39) than for diploid (5.5, n = 75) C-ALL patients (p < 0.002), even if the residual diploid G0/G1-phase cells were included in the calculation with the hyperdiploid G0/G1-phase cells. In the hyperdiploid group, the percentage of S-phase cells was not correlated with the amount of increase in ploidy (r = 0.05).

In the ALL group, the median percentage of S-phase cells was significantly higher for the L3 FAB morphological group (p < 0.001), corresponding to the finding for the B-ALL phenotype. The median percentage of S-phase cells was similar for the L1 (6.6, n = 129) and L2 (7.2, n = 32) FAB groups. In the ANLL group, there was no significant difference in median percentage of S-phase cells by FAB categories.

**Multiple Leukemia Stem Lines**

Two or more leukemia stem lines were evident by FCM in 23 patients with ALL (13.3%) and 5 with ANLL (9.6%) (Table 2). In 26 patients, the second stem line was in the G1+M region of the primary stem line, and the excess of cells in the G1+M region of the histogram was evident from the 4N1 (Fig. 1). The group includes patients 7 and 26 with near-haploid ALL and ANLL, respectively. The remainder had primary stem lines with modal numbers of 45–52 chromosomes. In the C-ALL group, a second stem line was more frequently associated with diploid primary stem lines than with FCM-detectable hyperdiploid.

<table>
<thead>
<tr>
<th>Pt. No.</th>
<th>Leukemia Phenotype</th>
<th>Percent of Blast§</th>
<th>Phases of Leukemia Stem Lines§</th>
<th>Dis of Leukemia Stem Lines</th>
<th>Percent of Cells 4N§ in G0/G1</th>
<th>4N§</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>C-ALL</td>
<td>97</td>
<td>46, +92</td>
<td>1.0, 1.98</td>
<td>95.5(2N). 2.2(4N)</td>
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<td>2</td>
<td>C-ALL</td>
<td>75</td>
<td>46P, 60</td>
<td>1.0, 1.31</td>
<td>50.0(2N). 42.1(2.6N)</td>
<td>7.5</td>
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<tr>
<td>3</td>
<td>T/E+ C. ALL</td>
<td>98</td>
<td>NA</td>
<td>1.0, 2.02</td>
<td>56.8(2N). 1.2 (4N)</td>
<td>3.5</td>
</tr>
<tr>
<td>4</td>
<td>U-ALL</td>
<td>99</td>
<td>46P, 92</td>
<td>1.0, 2.04</td>
<td>39.8(2N). 53.5(4N)</td>
<td>10.6</td>
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<td>C-ALL</td>
<td>99</td>
<td>46, +1</td>
<td>1.0, 2.04</td>
<td>97.5(2N). 0.9(4N)</td>
<td>2.8</td>
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<td>6</td>
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<td>86</td>
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<td>1.0, 2.17</td>
<td>69.9(2N). 22.9(3.4N)</td>
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<td>95</td>
<td>28, 56</td>
<td>0.6, 1.20</td>
<td>2.1(2N). 94.6(2.4N)</td>
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<td>C-ALL</td>
<td>99</td>
<td>47, 49, +92</td>
<td>1.0, 1.98</td>
<td>96.1(2N). 1.2(4N)</td>
<td>3.1</td>
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<tr>
<td>9</td>
<td>C-ALL</td>
<td>91</td>
<td>52, +92</td>
<td>1.0, 2.0</td>
<td>77.0(2N). 16.2(4N)</td>
<td>4.9</td>
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<tr>
<td>10</td>
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<td>99</td>
<td>49, +98</td>
<td>1.0, 2.08</td>
<td>90.6(2N). 3.6(4N)</td>
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<td>11</td>
<td>C-ALL</td>
<td>96</td>
<td>45, 90, 109</td>
<td>1.0, 1.95, 2.49</td>
<td>89.2(2N). 5.1(3.9N). 2.5(5N)</td>
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<tr>
<td>12</td>
<td>C-ALL</td>
<td>96</td>
<td>47, +92</td>
<td>1.0, 2.07</td>
<td>89.2(2N). 6.2(4.1N)</td>
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<tr>
<td>13</td>
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<td>47, +92</td>
<td>1.0, 1.93</td>
<td>90.0(2N). 2.6(3.9N)</td>
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<td>14</td>
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<tr>
<td>15</td>
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<td>93.3(2N). 1.8(4N)</td>
<td>5.5</td>
</tr>
<tr>
<td>16</td>
<td>C-ALL</td>
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<td>NA</td>
<td>1.0, 1.91</td>
<td>99.1(2N). 0.3(3.8N)</td>
<td>6.2</td>
</tr>
<tr>
<td>17</td>
<td>C-ALL</td>
<td>93</td>
<td>46, +92</td>
<td>1.0, 1.78, 2.02</td>
<td>66.9(2N). 9.4(3.6N). 20.4(4N)</td>
<td>87.7</td>
</tr>
<tr>
<td>18</td>
<td>C-ALL</td>
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<td>46P, +92</td>
<td>1.0, 1.97</td>
<td>94.5(2N). 0.9(3.9N)</td>
<td>4.4</td>
</tr>
<tr>
<td>19</td>
<td>ALL</td>
<td>95</td>
<td>NA</td>
<td>1.0, 1.23</td>
<td>60.0(2N). 35.8(3.2N)</td>
<td>1.2</td>
</tr>
<tr>
<td>20</td>
<td>C-ALL</td>
<td>95</td>
<td>47, +92</td>
<td>1.0, 1.97</td>
<td>89.7(2N). 2.4(3.9N)</td>
<td>4.7</td>
</tr>
<tr>
<td>21</td>
<td>C-ALL</td>
<td>97</td>
<td>46, +92</td>
<td>1.0, 1.98</td>
<td>88.9(2N). 2.2(4N)</td>
<td>4.8</td>
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<tr>
<td>22</td>
<td>C-ALL</td>
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<td>45, +92</td>
<td>1.0, 1.95</td>
<td>83.2(2N). 5.4(3.9N)</td>
<td>6.8</td>
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<tr>
<td>23</td>
<td>C-ALL</td>
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<td>46P, +92</td>
<td>1.0, 2.0</td>
<td>89.8(2N). 0.5(4N)</td>
<td>4.8</td>
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<tr>
<td>24</td>
<td>ANLL</td>
<td>78</td>
<td>NA</td>
<td>1.0, 2.13</td>
<td>49.0(2N). 48.0(4.3N)</td>
<td>75.1</td>
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<tr>
<td>25</td>
<td>ANLL</td>
<td>99</td>
<td>NA</td>
<td>1.0, 1.90</td>
<td>74.9(2N). 7.6(3.8N)</td>
<td>5.6</td>
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<tr>
<td>26</td>
<td>ANLL</td>
<td>89</td>
<td>NA, 46P</td>
<td>0.5, 1.0</td>
<td>1.0(1N). 92.4(2N)</td>
<td>655.4</td>
</tr>
<tr>
<td>27</td>
<td>ANLL</td>
<td>89</td>
<td>46P, 92</td>
<td>1.0, 1.99</td>
<td>89.5(2N). 1.9(4N)</td>
<td>4.3</td>
</tr>
<tr>
<td>28</td>
<td>ANLL</td>
<td>94</td>
<td>46P, 92</td>
<td>1.0, 1.98</td>
<td>56.3(2N). 13.4(4N)</td>
<td>7.0</td>
</tr>
</tbody>
</table>

All phenotypes as defined in Table 1; ANLL, acute nonlymphoblastic leukemia; P, pseudodiploid; N, human haploid number; §Patients 1–11 and 24–25 were studied with the TPS-1 and patients 12–23 and 26–28 with the EPICS-V flow cytometers (see Methods).

All metaphases were not found to correspond with the DI of 2.04 (pt. 5), DI of 1.78 (pt. 17), and DI of 0.5 (pt. 26).

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mia is easily detected by FCM because these cells are represented in a region of the histogram with very low background.29

The histogram from patient 4 shows pseudodiploid and tetraploid stem lines at diagnosis (Fig. 6). Cells from both stem lines were progressing through the cell cycle, since S-phase cells for each were evident. At relapse 16 mo later, only the pseudodiploid stem line was evident by karyotype and FCM analysis (Fig. 6B). Patient 8 had three leukemia stem lines at diagnosis, and at relapse, the tetraploid and hypertetraploid stem lines were not found by karyotype or FCM analysis (Fig. 7). In the karyotype at relapse, a new pseudodiploid stem line and the former 45-chromosome stem line were evident.

DISCUSSION

The clonal origin of human leukemia is reflected in
the uniformity of glucose-6-phosphate dehydrogenase isotypes,30 surface differentiation antigens,31 marker chromosomes,32 and DNA content7 in the cell population from individual patients. In our study, clonal abnormalities of leukemia cell DNA content were detected by FCM in 54 of 225 children. Based on the correlation between the DI and the karyotype modal chromosome number, the detection of an abnormal DI primarily depends on gains or losses of whole chromosomes of average size. The low dispersion (CV) of G0/G1 DNA content in leukemia stem lines supports the concept of a clonal abnormality of DNA complement in the malignant cells7 and confirms results for adult leukemia by Barlogie and coworkers.8

With the improved resolution of the EPICS-V instrument, stem lines with as few as 2 extra chromosomes per cell can be detected, although ≥3 extra chromosomes per cell are needed for consistent detection. Therefore, samples that were diploid by FCM analysis included those that were normal, pseudodiploid, hyperdiploid 47 or 48, and hypodiploid 44 or 45. The difference in the detectability of aneuploidy may also depend on the chromosomes involved, since different chromosomes have detectable differences in DNA content.33 Aberrant fluorochrome binding by malignant cell DNA is another factor that may create differences between karyotypic and flow cytometric measurements of DNA per cell.10 One of our cases with B-cell leukemia with an abnormal DI of 1.07 and a pseudodiploid karyotype may belong to this category. Alternatively, the abnormal DI in this case may have resulted from propidium-iodide staining of RNA as well as DNA.

FCM determination of DNA content complements karyotype analysis, since the DI can be estimated even when metaphases are in low proportion to other cells. Chromosome numbers per metaphase often show a wide range, partly because of cell fragility and loss during preparation. For this reason, criteria have been proposed by the International Workshop on Chromosomes in Leukemia to designate true abnormal stem lines.34 For multiple stem lines, DNA FCM can help to identify the abnormal populations that can often be confirmed by careful cytogenetic analysis.9

C-ALL antigen-positive lymphoblasts had a higher frequency of aneuploidy by FCM than did antigen-negative lymphoblasts (p < 0.003) or ANLL blasts (p < 0.001). The more differentiated T- and B-cell ALL cases in this study rarely had FCM-detectable aneuploidy. Nevertheless, abnormal karyotypes were equally frequent in T- and C-ALL because the T-cell group tended to be pseudodiploid or have small numerical changes that were undetectable by DNA FCM analysis.35 The Third International Workshop on Chromosomes in Leukemia has reported a similar frequency of hyperdiploidy in childhood non-T, non-B ALL (33%) and a similar absence of hyperdiploidy in T- or B-ALL.36

The associations between abnormal DNA content and differentiation antigens expressed by the leukemia cells may be related to the process of malignant transformation. For example, the increased frequency of hyperdiploidy in C-ALL could mean (1) that lymphoid progenitor cells with this phenotype are more susceptible to transformations that produce hyperdiploidy, or (2) that hyperdiploid leukemia clones that arise in pluripotent stem cells cannot differentiate beyond the C-ALL phenotypic stage due to the hyperdiploid chromosomal abnormality.

A second stem line was evident by FCM in 28 of 225 patients in this study. In 26 cases, the second stem line had about twice the DNA content of the primary stem line, a finding confirmed by cytogenetic analysis in 17 of these patients. The second stem line was proven to derive from a doubling of the primary stem line in all 4 assessable cases by documenting the duplication of extra whole chromosomes or abnormal markers in the karyotype.

Clonal evolution of leukemia karyotypes, as typified by chronic myelogenous leukemia, usually occurs by the acquisition of one or a few numerical or structural changes in subpopulations of cells.32,37 More radical clonal evolution that produces polyploidy could involve endoreduplication or other abnormalities of mitosis.38,39 While it is not unusual to see polyploid cells in ALL,40 our findings suggest that the frequency of two functionally independent but genetically related stem lines may be greater than is currently recognized. For three patients with multiple stem lines at diagnosis, DNA histograms and chromosome analysis at relapse showed the pseudodiploid and near-diploid stem lines without the polyploid stem lines. Further cases will be required to confirm that the stem line with the lower ploidy is more likely to become drug resistant and recur during therapy.

The percentage of cells in S-phase was significantly higher for patients with the B- and T-cell ALL phenotypes, thus confirming other published studies.14-16 The increased percentage of S-phase cells in the T-cell group was confined to those expressing heat-stable E-rosette receptors, as also reported by Dow and coworkers for a separate group of patients for whom labeling indices were determined.15 The pattern of percentage of S-phase cells reflects a hierarchy related to the intrinsically state of neoplastic lymphoid differentiation (B-cell > T-cell > non-T, non-B) in ALL as well as in non-Hodgkin's lymphoma, and probably mirrors the growth characteristics of the normal lymphoid
counterparts.\textsuperscript{17} Since both aneuploidy and S-phase cell percentage varied according to leukemia cell phenotype, we assessed their relationship in the C-ALL group and found that the median percentage of S-phase cells in hyperdiploid cases exceeded that in diploid cases. This is similar to findings in human and canine solid tumors,\textsuperscript{7,41} although the degree of hyperdiploidy in C-ALL did not correlate with the S-phase percentage. The failure to demonstrate a correlation may be due in part to the high degree of clustering of hyperdiploid DI values around 1.2 in C-ALL.

Differences in the median percentages of S-phase cells in diploid and hyperdiploid C-ALL may reflect a difference in the proliferation rate or the S-phase duration (or both).\textsuperscript{42} Assuming that the DNA synthetic rate is relatively constant, the S-phase durations of diploid leukemias would be similar and the percentage of cells in S phase would depend on the proliferative rate. Hyperdiploid leukemias, however, would require more time to duplicate the excess genetic material. Thus, the increased median percentage of S-phase cells in hyperdiploid compared to diploid C-ALL may reflect a longer S-phase duration rather than a higher proliferative rate. This distinction could have therapeutic importance, since an increased proliferative rate may predispose to the early emergence of drug-resistant mutants,\textsuperscript{43} whereas an increased S-phase duration in hyperdiploid cases may make the cells more vulnerable by prolonging the period of maximum drug sensitivity.

The influence of ploidy in ALL assessed by karyotype has been analyzed for prognostic significance. Based on our analysis,\textsuperscript{35} the results reported by Secker-Walker et al.,\textsuperscript{44} and the Third International Workshop on Chromosomes in Leukemia,\textsuperscript{46} hyperdiploidy (>50 chromosomes per cell) is correlated with a longer duration of complete remission in ALL. DNA analysis by FCM is an ideal way to detect this favorable prognostic group, since it rapidly provides a DI that is independent of the mitotic index and reliably correlated with the karyotype for hyperdiploid (>50) stem lines. Karyotypic analysis is a needed complement to the FCM method to identify the more subtle structural and numerical abnormalities that may also be prognostically important.\textsuperscript{34}

The S-phase cell percentage has also been linked to outcome in childhood ALL, with a significantly shorter median remission in the group with >6% S-phase cells.\textsuperscript{15,46} Since we have found that hyperdiploid C-ALL patients have a higher median S-phase percentage than do diploid patients, there is an apparent contradiction between the effect of these two variables. Further clinical follow-up and multivariate analysis of our cases may clarify the combined influence of ploidy and S-phase cell percentage on the outcome of treatment in childhood ALL.

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Aneuploidy and percentage of S-phase cells determined by flow cytometry correlate with cell phenotype in childhood acute leukemia

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