Interrelationship of Immunologic Characteristics, Proliferation Pattern, and Prednisone Sensitivity in Acute Lymphoblastic Leukemia of Childhood

By J. A. J. M. Bakkeren, G. A. M. de Vaan, and H. F. P. Hillen

In children with acute lymphoblastic leukemia (ALL) the effect of prednisone therapy on the cell-cycle phase distribution of leukemic bone marrow cells was determined with pulse cytophotometry at the time of diagnosis. Also, the interrelationship with the presence of the sheep erythrocyte receptor as a marker for T cells was investigated. In 17 of 21 patients prednisone treatment caused a decrease in the percentage of cells in the $S + G_2 + M$ phases. In 11 of 12 adult patients with ALL the same result was obtained. In a group of 31 children with ALL, 8 cases of T-cell ALL occurred in combination with intermediate or high WBC counts. The 3 patients with the highest E-rosetting percentages had rather high proportions of bone marrow cells in the $S + G_2 + M$ phases. No correlation could be established between the WBC count or the E-rosetting percentage and the chance for complete remission.

It is common experience that the length of the first remission of acute lymphoblastic leukemia (ALL) is usually much longer in children than in adolescents and adults. In both age groups, however, a rather similar rate of complete remission can be obtained by treatment with prednisone and vincristine. Nevertheless, the nature of a biologic difference between ALL in children and ALL in adults is not known.

The influence of the prednisone therapy on the proliferation pattern of the leukemic bone marrow cells from adults suffering from ALL was studied by one of us. In a pulse cytophotometric study very definite decreases in the proportions of cells in the $S$ and $G_2 + M$ phases were demonstrated after 3 days of oral prednisone therapy. This mode of therapy had no effect on bone marrow proliferation in patients with acute myeloid leukemia (AML) or in patients with normal bone marrow morphology. It was thought worthwhile to study in children with ALL the effect of prednisone therapy on the proliferative behavior of the leukemic bone marrow cells.

The duration of a complete remission in ALL in childhood is negatively correlated with a high initial white blood cell (WBC) count and with the presence of T-lymphocyte markers on the leukemic cells.

In a previous study the immunologic markers and functions of leukemic lymphoblasts were determined in a large series of children with ALL before any treatment. In the present report this series is enlarged and extended with a pulse cytophotometric examination of the proliferation pattern of the bone marrow and the influence of prednisone thereon. Assuming that prednisone sensitivity of the
bone marrow leukemic cells and absence of the prognostically bad features for ALL of childhood (a high WBC count and the presence of T-lymphocyte markers on the leukemic cells) are favorable conditions for a longer duration of remission, we sought a possible interrelationship between the proliferation pattern and the prognostically bad parameters. Therefore, we determined in children with newly diagnosed ALL the presence of immunologic T- and B-cell markers on their leukemic cells and correlated these data with the initial WBC count and the proliferation pattern of the bone marrow leukemic cells. For comparison, pulse cytophotometric and clinical data of adult patients with ALL are included.

MATERIALS AND METHODS

Patients

In this study 36 children with ALL (ages between 0.5 and 15 yr) and 13 adults with ALL (ages between 15 and 68 yr) were investigated at the time of diagnosis. Eight children (ages between 4 and 15 yr) suffering from leukemic transformation of non-Hodgkin malignant lymphoma were also included in the study. In all cases of ALL the diagnosis was obtained on bone marrow aspirates. The diagnosis was confirmed, for adults, at the World Health Organization Reference Centre for Leukemias, Villejuif, France, and, for children, at the Reference Centre of the Dutch Working Group on Leukemia in Children (NWLK), The Hague, The Netherlands. The diagnosis of leukemic conversion of non-Hodgkin malignant lymphoma was also made by inspection of bone marrow slides but was not confirmed by any outside reference center.

In the group of children with ALL some preselection was inevitable because a greater proportion of patients who were classified as bad risks because of initial WBC counts greater than 50,000/μl were sent by pediatricians in local clinics to the Children’s Oncological Centre of the Radboud Hospital for more advanced treatment. There was no such bias in the group of adult patients with ALL nor in the group of children with non-Hodgkin malignant lymphoma with leukemic conversion.

Pulse Cytophotometric Studies

For DNA measurement 0.2-0.5 ml of a bone marrow aspirate was mixed with 0.5 ml of buffered ACD as an anticoagulant. A small volume of the aspirate containing approximately 10⁶ nucleated cells was added dropwise to a hypotonic staining solution with 25 μl of ethidium bromide dissolved in 0.1% sodium citrate. Before measurement the sample was filtered through a nylon filter (100-μ mesh). After 20 min staining at room temperature the bone marrow samples were measured in an ICP-II (Phywe, Göttingen, West Germany). The DNA partition of 5-10 × 10⁶ cells was recorded in a histogram. The percentages of cells in G₁, S, and G₁+M phases were calculated from the histograms by a computerized method.

Immunologic Studies

The immunologic studies reported here were performed only in almost completely homogeneous (≥95%) leukemic cell populations from bone marrow and/or peripheral blood.

Cell Separation

The leukemic cells were isolated from peripheral blood by the Ficoll-Isopaque gradient centrifugation method. After two washings, 4 × 10⁶ cells were resuspended in 1 ml of 0.25-M Tris-buffered minimum essential medium (MEM) containing 5% pooled human serum.

For the isolation of leukemic cells from bone marrow, 0.5 ml of bone marrow aspirate was collected into phosphate-buffered saline (PBS) with 5% EDTA. After mixing with 1 ml of Tris-buffered MEM containing 20% pooled serum, the cell suspension was layered on 1 ml of Ficoll-Isopaque and centrifuged. For rosette testing 4 × 10⁶ cells were resuspended after two washings in 1 ml of 0.25-M Tris-buffered MEM containing 5% pooled human serum. For immunofluorescence studies the cells in the interface after centrifugation on Ficoll-Isopaque were washed twice with PBS containing 1% bovine serum albumin (BSA) at 4°C. Thereafter, 10⁶ cells were resuspended in 50 μl of the same solution.
E-Rosetting Test

The E-rosetting test was performed according to Stjernswärd as reported earlier. Briefly, lymphocytes were separated from peripheral blood or bone marrow by the Ficoll-Isopaque gradient centrifugation method and washed. Lymphocytes (10^6) in 0.25-M Tris-buffered MEM containing 5% human serum were mixed with 0.25 ml of a 1% suspension of sheep erythrocytes. After incubation at 37°C for 15 min the cell mixture was centrifuged for 2 min at 200 g at 4°C. The cells were kept at 4°C overnight, then carefully resuspended, and the rosettes were counted.

Immunofluorescence Studies

Cell suspension (50 µl or 10^6 cells) in PBS with 1% BSA was mixed with 50 µl of fluorochrome-labeled antisera [antihuman Ig conjugated with tetramethyl rhodamine isothiocyanate (TRITC) supplied by Nordic Immunological Laboratories, Tilburg, Holland] and 50 µl of PBS with 1% BSA. Incubation was performed at 4°C during 30 min. After incubation the cells were washed twice with the 1% BSA solution and placed on a slide with one drop of buffered glycerol. The slides were examined under a Zeiss Standard microscope equipped with a Ploem-type vertical illuminator, a high-pressure mercury lamp HBO 50, and a suitable filter combination.

RESULTS

Pulse Cytophotometric Studies

At the time of diagnosis and before any treatment, pulse cytophotometric (PCP) histograms of bone marrow aspirates were obtained from 26 children with ALL, and the proportions of cells in the S and G2+M phases were calculated. In 2 children no calculations of the percentages of cells in the different cell-cycle phases could be performed because of the presence of cells with an abnormal DNA content. In 21 children these investigations could be repeated after 3 days of treatment with prednisone only (40 mg/sq m once daily before noon, orally). In 3 patients not enough bone marrow aspirate could be obtained after 3 days of prednisone. The results are depicted in Fig 1. The mean percentage of cells in the S+G2+M phases was, for the group of children taken as a whole, significantly lower (p < 0.01) after prednisone treatment (arithmetic means 10.7 and 6.6, respectively). The initial S+G2+M percentages varied widely between 2% and 23%. The decrease observed after prednisone treatment also varied widely, both absolutely and relatively. A decrease was observed in 17 of 21 cases. In 4 children no decrease was seen. Three of these 4 patients did not experience complete remission on vincristine and prednisone. With additional therapy (L-asparaginase for 2 wk in 2 patients and rubidomycine in 1 patient) a first complete remission was obtained. The fourth patient, a 5-yr-old girl, contracted a nearly fatal interstitial pneumonia in the first week of remission induction; however, she attained complete remission after treatment, during 5 wk, with only vincristine and prednisone. Of 17 children who showed decreases in S+G2+M percentages, 14 attained complete remission (Table 1). For 2 patients additional therapy was necessary. In 13 adult patients with ALL the same investigations were performed (Fig. 1). One of the patients had a leukemic cell population with abnormal DNA content and has therefore not been included in Fig. 1. Results similar to those in the children with ALL were obtained. With one exception, after prednisone treatment a consistent decrease in pretreatment S+G2+M percentages was found. Also, in adults, the mean percentage of cells in S+G2+M phases was significantly lower (p < 0.01) after prednisone treatment (arithmetic means 14.8 and 7.4, respectively). The S+G2+M percentages showed similar variability in adults and children. For the
group of 24 children as a whole, however, the mean percentage of cells in the S+G2+M phases was significantly lower ($p < 0.05$) than for the group of 12 adults (arithmetic means 10.7 and 14.8 respectively).

In Fig. 2 the proportions of bone marrow cells in the S+G2+M phases, as calculated from the histograms obtained before prednisone treatment, are shown in

<table>
<thead>
<tr>
<th>WBC (cells/liter)</th>
<th>Prednisone Sensitivity</th>
<th>Number of Patients</th>
<th>Abnormal DNA Content</th>
<th>Result</th>
</tr>
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<tbody>
<tr>
<td>Children</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>$&lt; 50 \times 10^9$</td>
<td>VCR + PRE</td>
<td>19</td>
<td>1</td>
<td>8</td>
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<td></td>
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<td>6 PR</td>
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</tr>
<tr>
<td></td>
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<td>3</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td></td>
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<td>2</td>
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<tr>
<td>Total</td>
<td></td>
<td>26</td>
<td>2</td>
<td>17</td>
</tr>
<tr>
<td>Adults</td>
<td>VCR + PRE</td>
<td>12</td>
<td>1</td>
<td>8</td>
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<td></td>
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<tr>
<td>Total</td>
<td></td>
<td>13</td>
<td>1</td>
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**Abbreviations:** RI, remission-induction; VCR, vincristine; PRE, prednisone; HN2, nitrogen mustard; Adria, adriamycin; CR, complete remission; PR, partial remission; F, failure; n.d., not done; +, decrease in percentage of leukemic bone marrow cells in S + G2 + M phases after 3 days of prednisone treatment; −, no decrease in percentage of leukemic bone marrow cells in S + G2 + M phases after 3 days of prednisone treatment.
relation to the WBC counts. In this figure the data from 24 children and 12 adults are combined. It appears that no clear correlation exists between the WBC count and the S+G2+M percentage. The values for the group of children and for the group of adult patients show similar patterns of variation between the two parameters; a large proliferating fraction in the bone marrow does not necessarily give rise to a high WBC count. In the group of children the two highest values for the S+G2+M percentage (>20%) were found in patients with rather low WBC counts (12.4 and 17.1 × 10^9 cells/liter, respectively).

**Relationship Between Pulse Cytophotometric and Immunologic Data**

The relationship between WBC count and E-rosetting capacity of leukemic cells for 31 children with ALL before any treatment is given in Fig. 3. It can be seen that four definite T-cell leukemias (E-rosetting percentage >30%) were found in the group of 10 patients with high WBC counts (>50 × 10^9 cells/liter). In the group of 21 patients with WBC counts less than 50 × 10^9 cells/liter, only two cases of T-cell leukemia were present, both showing rather intermediate values of E-rosetting (33% and 44%, respectively). It is remarkable that among newly diagnosed children with ALL the 3 patients with the highest E-rosetting percentages (>35%) had rather high proportions of cells in the S and G2+M phases (Fig. 4). From the moment in our series of investigations where immunofluorescence studies became possible, in 12 children also the proportions of surface-Ig-bearing lymphoid cells were determined. No cases with a high percentage of this lymphocyte subclass were detected.

Determination of the immunologic parameters could be repeated after prednisone treatment in 9 patients. In these cases no clear differences appeared to exist between the percentages of E-rosetting cells obtained before and after treatment.

In Table 1 the mode of remission-induction therapy and the clinical course of the
disease are summarized for 26 children and 13 adults with ALL. In addition, the effects of prednisone treatment on the proliferating bone marrow cells are tabulated for the different patient groups. In 5 children, all of them having high peripheral WBC counts at presentation, the remission-induction therapy with vincristine and prednisone, after the first 3 days of treatment with prednisone alone, was combined with other cytostatics. In the other 21 children the results of vincristine-prednisone alone could be evaluated. As the rate of successful remission induction was high in the whole group of patients, no definite correlations can be established between initial WBC count, E-rosetting percentage, pretreatment S + G2 + M percentage, or decrease of the latter under the influence of prednisone, on the one hand, and the chance for obtaining complete remission, on the other.
hand. Also, in the adult patients, no correlation seems to exist between the results of the remission-induction treatment, on the one hand, and initial WBC count, proliferating fraction of bone marrow, or its change after prednisone treatment, on the other hand. A rather consistent feature is, in both age groups, the fall in WBC count, especially in cases with high initial WBC counts.

A few patients (8 children) with leukemic transformation of non-Hodgkin malignant lymphoma were investigated in the same way as the ALL patients for extent of the proliferating fraction in the bone marrow and the influence of prednisone treatment thereon. In 3 children sufficient bone marrow samples could not be obtained after the 3 days of prednisone treatment. In two cases the second tap was dry, whereas 1 patient died 24 hr after the first prednisone dose. In this group of 8 children with non-Hodgkin malignant lymphoma the mean percentage of bone marrow cells in the S+G2+M phases was higher (19.4%) than in the group of childhood ALL (10.7%). Prednisone treatment generally did not appear to diminish the percentages of bone marrow cells in the S+G2+M phases in these patients. However, the group of patients with non-Hodgkin malignant lymphoma is too small (8 children) to warrant any definite conclusion.

DISCUSSION

Usually the first remission of ALL lasts much longer in children than in adolescents and adults, whereas the rates of remission induction with prednisone and vincristine are rather similar in these age groups.

Since in an earlier study\(^1\) it was found that after prednisone treatment there was a decrease in the proliferating fraction of the bone marrow in adult patients with ALL, it seemed to us worthwhile to investigate the cell kinetic effects of prednisone in both children and adults with ALL. Therefore, the first objectives of our study were to measure the percentages of proliferating bone marrow cells before treatment and to compare the influences of prednisone treatment on these percentages in both children and adult patients with ALL. In childhood ALL, prognostically bad parameters are a high WBC count and the presence of T-lymphocyte markers on the leukemic cells. Another objective of our study was to search for a possible interrelationship between these two parameters and the size of the proliferating fraction in the bone marrow and the effect of prednisone treatment thereon in children with ALL.

In the children with ALL described in this report, the proportion of proliferative cells in the bone marrow generally decreased after 3 days of treatment with prednisone. It appears that in newly diagnosed ALL of childhood the same prednisone sensitivity exists as in ALL of adults. For both age groups it was established that a significant difference exists between the mean percentages of S+G2+M phases before and after prednisone treatment. Moreover, there seems to exist no preferential effect of prednisone on either T-cell or “null-cell” ALL of childhood. The WBC counts diminished in both age groups after prednisone treatment, at least in the cases with high initial leukocyte counts. A remarkable finding in our series of children and adult patients seems to be the lower pretreatment proliferating fraction in the bone marrow in children as compared with adult patients.

In 2 children and 1 adult patient it was not possible to calculate the percentages of leukemic bone marrow cells in the different phases of the cell cycle. Most
probably this is a consequence of an extreme aneuploidy, which often is found in chromosomal investigations of ALL. Rowley stated in a recent review that approximately 50% of patients with ALL have a normal karyotype, the others being mostly hyperdiploid. In our series of 45 patients we found 3 patients with strongly deviating DNA content. Therefore we must assume that among the other patients several cases occur with an aneuploidy that cannot be detected by the pulse cytophotometric method employed. For an exact proof of aneuploidy besides pulse cytophotometric studies, karyotyping must be done.

Increasing numbers of investigators are reporting that in about one-fifth of all ALL cases the leukemic cells possess E-rosetting capacity. These cases are generally qualified as T-cell ALL. In the series of children with ALL described in this communication, about 20% of all cases could also be qualified as T-cell ALL on the basis of E-rosette formation.

Our data are in accordance with the general belief that T-cell ALL is associated with a high WBC count. To examine the possibility that a high WBC count is reflected by a high proliferating fraction in the bone marrow, the relationship was studied between E-rosetting capacity and proportion of proliferating cells in the bone marrow, expressed as percentages of cells in the S and G2 + M phases. Our results point to a positive correlation between the two parameters. Patients with T-cell leukemia generally seem to possess a somewhat larger proliferating pool than patients with non-T-cell leukemia. Such a correlation was suggested from the experiments carried out by Schwenk and Schneider in an in vitro study with a permanently growing lymphoblastic cell line established from the leukemic lymphoblasts of a child with T-cell ALL. The authors showed that E-rosetting capacity was reduced in cells in the G1 phase and thereupon concluded that lymphoblasts with T-cell markers on their surfaces represent a rapidly proliferating cell population. In contrast to these findings is the observation of Arlin et al., who in studying an adult patient with T-cell ALL found evidence that the expression of the sheep erythrocyte receptor is independent of both the phase of the cell cycle and the morphologic appearance of the leukemic cells. In view of these conflicting results, it is clear that more work must be done to answer the question whether the leukemic cells from T-cell ALL are a more rapidly proliferating population than the cells from non-T-cell ALL.

Murphy et al. carried out, in a large group of children with ALL, investigations on the pretreatment bone marrow mitotic index (MI) and in vitro 3H-thymidine labeling index (LI). These authors, too, reported a very broad range of values for both parameters. No correlation was found among MI, LI, and initial WBC count. These findings are not in agreement with the report of Hart et al., who found a significant correlation between bone marrow LI and absolute number of blasts in peripheral blood in adult patients with ALL. Furthermore, Murphy et al. found that their so-called E-positive patients had significantly higher initial LIs and MIs than their E-negative group. This is in good agreement with the relationship indicated by our experiments, between E-rosetting capacity and proportion of the proliferative fraction in the bone marrow.

A very important point arising from our study seems to be that in searching for an explanation for the well-known differences in remission duration and cure percentage between children and adults with ALL, it is unlikely to be fruitful to seek a difference in prednisone sensitivity, as in this respect children and adults do
not differ. It is not excluded that the difference could be found in the lower pretreatment proliferating fraction in the bone marrow found in our series of children, as compared with the series of adult patients.

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