Sequential Surface Marker and Cytokinetic Studies on Individual Cells From Children With Acute Lymphoid Leukemia

By H. P. Wagner and A. Hirt

Sequential immunological and cytokinetic studies were done on individual lymphoid cells in the peripheral blood of 10 children with previously untreated acute lymphoid leukemia (8 non-T-non-B, 1 T, and 1 B-ALL) and on lymphoid bone marrow cells (4 non-T-non-B ALL). The results obtained suggest that some, but not all, of the children considered to have non-T-non-B ALL had proliferating leukemic cells with surface immunoglobulin (SIg) in the peripheral blood. The absolute number of these SIg+ proliferating leukemic cells, however, was always smaller than the absolute number of SIg- proliferating leukemic cells. A similar observation was made in the child with B-ALL. On the other hand, in the blood of the child with T-ALL, no SIg+ proliferating leukemic cells were detected. The results indicate that sequential analyses of different parameters on single cells might permit a more precise investigation of relations between cell proliferation and cell differentiation or of interactions between normal and neoplastic lymphoid cells.

In the past, cytokinetic studies of bone marrow cells from children with acute lymphoid leukemia (ALL) revealed variable proliferative patterns but failed to be of prognostic significance. More recently, the assessment of surface markers became an important tool for the indentification and enumeration of different types of lymphocytes. Abnormal distributions of surface marker gave further evidence for the heterogeneity of ALL and proved to be of prognostic significance. Parallel cytokinetic and surface marker studies indicated that the labeling index, the mitotic index, or the percentage of bone marrow cells in the S, G2, and M phases of the cell cycle were higher in T than in non-T-non-B ALL. To our knowledge, only preliminary results of sequential surface marker and cytokinetic studies have been published on individual lymphoid blood and bone marrow cells of patients with ALL. We therefore report our investigations of 10 children with previously untreated ALL.

Materials

Patients

Lymphoid cells (normal and neoplastic) in the blood (bone marrow) of 10 children with previously untreated ALL were studied. In all patients the diagnosis was based on conventional morphological and cytochemical studies of bone marrow aspirates.

Five patients had low initial white blood cell counts (table I). In two of the five (T.B. and C.J.) too few bone marrow cells were obtained for marker studies. Of the remaining three, patient F.B. had 2% B cells and 3% T cells in the bone marrow; for patient H.U., the corresponding numbers were 2% and <1%, respectively; in the bone marrow of patient D.C. 9% B cells were found. The relative number of T cells could not be determined.

Although, for reasons discussed below, an investigation of lymphoid cells found in the blood appeared more rewarding than a study of bone marrow cells, parallel studies on lymphoid cells in blood and bone marrow were done in 4 patients (Table I). Age, sex, survival, initial white blood cell count, and the percentage of lymphoid cells in the peripheral blood (bone marrow) are shown in Table I. The first eight patients were considered to have non-T-non-B ALL, patient F.J. B-ALL, and patient M.N. T-ALL.

All studies were done with the approval of our human studies committee in accordance with the declaration of Helsinki.

Sequential Surface Ig (Slg) and Cytokinetic Studies

One milliliter EDTA (5% in 0.01 M PBS, pH adjusted to 7.8 with 10 M NaOH) was added to either 9 ml blood or 1-2 ml of bone marrow. Tritiated thymidine (3HdT, New England Nuclear, specific activity 6.7 Ci/mM, 10 µCi/ml) was added to obtain a final activity of 1 µCi/ml. After 30 min at 37°C, the pulse-labeling was stopped with an equal volume of cold (4°C) PBS. The mononuclear cells were isolated with Ficoll (Pharmacia Fine Chemicals) - Rompac 440 (Nyegaard), washed twice (300g for 10 min) at 4°C with PBS, and resuspended in 1 ml of washing solution (i.e., 10 ml 5% EDTA; 10 ml 22% bovine serum albumin; 1 M NaOH) was added to either 9 ml blood or 1-2 ml of bone marrow. Tritiated thymidine (3HdT, New England Nuclear, specific activity 6.7 Ci/mM, 10 µCi/ml) was added to obtain a final activity of 1 µCi/ml. After 30 min at 37°C, the pulse-labeling was stopped with an equal volume of cold (4°C) PBS. The mononuclear cells were isolated with Ficoll (Pharmacia Fine Chemicals) - Rompac 440 (Nyegaard), washed twice (300g for 10 min) at 4°C with PBS (0.01 M, pH adjusted to 7.8), resuspended in 1 ml of washing solution (i.e., 10 ml 5% EDTA; 10 ml 22% bovine serum albumin; PBS 0.01 M ad 100; pH adjusted to 7 with 5 M NaOH), and counted in order to avoid cell numbers greater than 10 x 10⁶/ml. After washing once more with washing solution at 4°C and resuspending in 0.1 ml of cold (4°C) washing solution, 0.2 ml of fluorescein isocyanate (FITC) conjugated anti-heavy-chain serum, together with tetramethylrhodamine isothiocyanate (TRITC) conjugated anti-light-chain serum, were added in a dilution of 1:20 and 1:10, respectively. The anti-heavy-chain serum was a mixture of specific but nonabsorbed sheep anti-IgG, anti-IgM, and anti-IgA; the anti-light-chain serum a mixture of specific sheep anti-λ sera. For one patient (F.J.) a specific FITC-conjugated anti-lgM and a specific TRITC-conjugated anti-lgA sheep serum were used in addition to the above. All antisera were conjugated as described by Hjimans et al. After incubation for 30 min at 4°C in the dark, the cells were washed twice with cold washing solution and resuspended in a small volume of the same solution. Brush smears were made with a Rowney's no. 1, series 40, sable brush. Air-dried smears were fixed in methanol for 30 min, air-dried, and mounted in fluoromount (Searle).

FITC fluorescence was used for localizing SIg+ cells, and

*0.01 M PBS: 170 g NaCl; 27.2 g KH₂PO₄; deionized water ad 2000 ml; pH adjusted to 7.8 with 10 N NaOH; before use diluted 1:10 with deionized water.

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Supported in part by the Swiss National Foundation for Scientific Research and by the Swiss Cancer League.

Submitted August 30, 1979; accepted June 4, 1980.

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0006-4971/80/5604-0012$01.00/0

Blood, Vol. 56, No. 4 (October), 1980
SURFACE AND CYTOKINETIC STUDIES IN ALL

Table 1. Age, Sex, Survival of Patients: Blood (Bone Marrow) Cell Counts; and Surface Marker and Cytokinetic Studies

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (yr) and Sex</th>
<th>WBC (x 10^3/μm)</th>
<th>Blast and Lymphoid Cells (%)</th>
<th>Slg+ Cells (%)</th>
<th>AET-SRBC Rosetting Cells (%)</th>
<th>Li of Blast and Lymphoid Cells (%)</th>
<th>Li of Slg+ Cells (%)</th>
<th>Li of Slg- Cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T.B.</td>
<td>7/4/12 δ</td>
<td>21+</td>
<td>2.3</td>
<td>20</td>
<td>70</td>
<td>0.8</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>C.J.</td>
<td>7/12 δ</td>
<td>20+</td>
<td>3.9</td>
<td>16</td>
<td>11</td>
<td>1.6</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td>F.B.</td>
<td>11 δ</td>
<td>15+</td>
<td>1.7</td>
<td>70</td>
<td>6</td>
<td>84</td>
<td>0</td>
<td>0.3</td>
</tr>
<tr>
<td>H.U.</td>
<td>3/12 δ</td>
<td>15+</td>
<td>2.7</td>
<td>97</td>
<td>5</td>
<td>54</td>
<td>0.2</td>
<td>0.9</td>
</tr>
<tr>
<td>D.C.C.</td>
<td>6/12 δ</td>
<td>13+</td>
<td>3.6</td>
<td>88</td>
<td>18</td>
<td>62</td>
<td>0.1</td>
<td>ND</td>
</tr>
<tr>
<td>G.St.</td>
<td>2 1/2 δ</td>
<td>14+</td>
<td>25</td>
<td>97</td>
<td>6</td>
<td>6</td>
<td>1.5</td>
<td>0.8</td>
</tr>
<tr>
<td>G.S.</td>
<td>1 4/12 δ</td>
<td>13+</td>
<td>16</td>
<td>71</td>
<td>20</td>
<td>5</td>
<td>5.9</td>
<td>1.1</td>
</tr>
<tr>
<td>B.R.</td>
<td>8 δ</td>
<td>12</td>
<td>100</td>
<td>87</td>
<td>3</td>
<td>3</td>
<td>16.7</td>
<td>5.9</td>
</tr>
<tr>
<td>F.J.</td>
<td>6/12 δ</td>
<td>7</td>
<td>35</td>
<td>40</td>
<td>73</td>
<td>9</td>
<td>9.2</td>
<td>5.5</td>
</tr>
<tr>
<td>M.N.</td>
<td>2 11/12 δ</td>
<td>10</td>
<td>127</td>
<td>70</td>
<td>3</td>
<td>90</td>
<td>ND</td>
<td>0</td>
</tr>
</tbody>
</table>

Li, labeling index.
Values in parentheses are those of bone marrow.

TRITC fluorescence was used to confirm the presence of Slg, particularly if fluorescence was weak. Two fluorescent probes were felt to be necessary, since the detection of surface fluorescence on fixed cells appeared to be more delicate than on cells in suspension. Practically no cells with only FITC or TRITC fluorescence were found.

Fluorescence studies were done with Zeiss microscope (Osram HBO 100 W/2 superpressure mercury lamp, filter, and beam splitter combinations; FITC: incident light: SP 490 and SP 500; beam splitter 510; barrier filter LP 520; TRITC: incident light BP 546/9, beam splitter 580, barrier filter LP 590). Slg+ cells were mapped with a computer-controlled (PDP-12, Digital, programmed with Apamos II, Zeiss) scanning table. After mapping a few hundred Slg+ and Slg- cells, the fluoromount was dissolved in xylol at room temperature until the cover glass fell off. The slides were rehydrated in ethanol (absolute, 80%, 70%, 60%, 40%, and 20%, 5 min each), rinsed in deionized water for 5–10 min, fixed in methanol for 30 min, and processed for radioautography using Kodak Nuclear Track Emulsion NTB-2 [exposure time: 4–5 days; development with D-19 (Kodak) for 2 min and fixation in Rapid Fixer (Kodak) for 5 min at 18–20°C; tap water, 10–30 min, at 18–20°C]. Autoradiographs were stained through the film by use of May-Grünwald (Merck) stain, diluted 1:5–1:10 with phosphate buffer according to Weise,13 pH 5.5 (staining time 10–20 min), rinsed for a few seconds in deionized water, and restained with May-Grünwald, diluted 1:5–1:10 with phosphate buffer according to Weise, pH 7.5 (staining time 5 min). After rinsing again the slides were destained, if necessary, with phosphate buffer according to Weise, pH 7.5 and finally air-dried.

On the May-Grünwald stained autoradiographs, the grains overlying the nuclei of previously mapped Slg+ and Slg- cells (see above) were registered. At least 1000 lymphoid cells were evaluated for the overall labeling index. Slg+ cells with monocytoid morphology were eliminated. Background corrections were based on the highest grain counts over cell-free areas of nuclear size. Interphase cells with four or more grains were regarded as being labeled.

In a final step, the slides were Feulgen-stained according to a standardized procedure described by Sordat et al.14 The nuclear DNA content of mapped cells was determined cytophotometrically as reported earlier.15

Determination of Slg+ and AET-SRBC-Rosetting Cells

The relative number of Slg+ cells was determined, with minor modifications, according to Preud'homme and Labaume.16 The relative number of cells forming rosettes with 2-S-amino-ethylisothiouronium bromide treated sheep red cells (AET-SRBC-rosetting cells) was estimated according to Kaplan, Woodson, and Clark.17 Our normal relative and absolute numbers of Slg+ and SRBC-rosetting cells corresponded well with the ranges indicated by Asma et al.18

RESULTS

The percentage of Slg+ and of AET-SRBC-rosetting lymphoid cells (normal and leukemic) are shown in Table 1. Also presented is the labeling index of all lymphoid cells and of surface-marker-defined subsets. The results of cytophotometric nuclear DNA determinations on Slg+ and Slg- cells are shown in Table 2.

Table 2. Cytophotometric DNA Measurements on Single Cells

| Patient | Material | Total Slg+ Cells | Slg+ Cells | G2/G1 | S | G2 | Total Slg- Cells | Slg- Cells | G2/G1 | S | G2 |
|---------|----------|-----------------|------------|-------|----|----|-----------------|------------|-------|----|----|----|
| T.B.    | Blood    | 488             | 100        | 488   | 100| 0  | 0               | 0          | 0     | 0  | 2 | 1 |
| C.J.    | Blood    | 240             | 100        | 236   | 98.3| 4  | 1.7             | 0          | 0     | 0  | 0  | 0 |
| B.R.    | Blood    | 178             | 100        | 165   | 92.7| 2  | 7.3             | 1          | 6.7   | 1  | 0.6|
| F.J.    | Blood    | 440             | 100        | 395   | 89.8| 23 | 5.2             | 22         | 5     | 22 | 5  | 22 |
| M.N.    | Blood    | 430             | 100        | 397   | 92.3| 23 | 7.7             | 9          | 2.3   | 9  | 2.3|

Table 2. Cytophotometric DNA Measurements on Single Cells

| Patient | Material | Total Slg+ Cells | Slg+ Cells | G2/G1 | S | G2 | Total Slg- Cells | Slg- Cells | G2/G1 | S | G2 |
|---------|----------|-----------------|------------|-------|----|----|-----------------|------------|-------|----|----|----|
| T.B.    | Blood    | 488             | 100        | 488   | 100| 0  | 0               | 0          | 0     | 0  | 2 | 1 |
| C.J.    | Blood    | 240             | 100        | 236   | 98.3| 4  | 1.7             | 0          | 0     | 0  | 0  | 0 |
| B.R.    | Blood    | 178             | 100        | 165   | 92.7| 2  | 7.3             | 1          | 6.7   | 1  | 0.6|
| F.J.    | Blood    | 440             | 100        | 395   | 89.8| 23 | 5.2             | 22         | 5     | 22 | 5  | 22 |
| M.N.    | Blood    | 430             | 100        | 397   | 92.3| 23 | 7.7             | 9          | 2.3   | 9  | 2.3|

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The absolute number of marker-defined subsets of lymphoid (normal and leukemic) blood cells is represented in Fig. 1 and the absolute number of labeled and unlabeled SIg+ and SIg− cells in Fig. 2.

Five patients considered to have non-T-non-B ALL (T.B., C.J., F.B., H.U., and D.C.C., Table 1) presented with a low initial white blood cell counts. Since it is difficult, at least for a fraction of lymphoid cells, to differentiate between normal and leukemic cells, no effort was made to separate these two groups of lymphoid cells. Nevertheless, the majority of the lymphoid cells in the blood of these five patients were small and morphologically similar to normal lymphocytes. In this group of patients the relative numbers of SIg+ and AET-SRBC-rosetting cells were within normal limits (Table 1). The absolute number of AET-SRBC cells (Fig. 1) was below normal in two (T.B., C.J.) and normal in three patients (F.B., H.U., D.C.C.). The absolute number of SIg+ cells was below normal in T.B. and F.B., at the lower limit of the normal range in C.J. and H.U., and normal in D.C.C.

The labeling index of peripheral blood lymphoid cells (normal and leukemic) in these five patients was low (Table 1) and practically all labeled cells appeared morphologically to be leukemic blasts. In the two patients in whom cytophotometric DNA measurements were made (T.B. and C.J., Table 2), SIg− cells in S were found, but only one (C.J.) had proliferating SIg+ cells. In patient F.B., the labeling indices of bone marrow cells were higher than those of blood cells. Both proliferating SIg− and SIg+ cells were found (Table 1). All of the five patients are in complete remission and have survived for 13 to 21+ mo (Table 1).

Three other children considered to have non-T-non-B ALL (G.St., G.S., B.R., Table 1) presented with an elevated initial WBC and with low relative number of AET-SRBC-rosetting cells. The absolute number of AET-SRBC cells (Fig. 1) was subnormal or normal, but the absolute number of nonrosetting SIg− and, to a lesser degree, of SIg+ cells was elevated. The labeling index of lymphoid (normal and leukemic) blood cells (Table 1) was higher than in the first group (Table 1). Again, practically all labeled cells were morphologically leukemic blasts. The labeling index of SIg− cells and also the absolute number of labeled SIg− cells were always higher than that of SIg+ cells (Table 1 and Fig. 2). Cytophotometric DNA measurements confirmed these labeling index data in B.R. They also indicated that in the bone marrow there was a larger fraction of G2 cells, particularly in the SIg− cells (Table 2). The patient with the highest labeling indices (B.R.) died in relapse 1 yr after diagnosis, whereas G.S. and G.St. with low labeling indices remain in complete remission 13+ and 14+ mo after diagnosis, respectively.

Patient F.J., considered to have B-ALL, had an elevated initial WBC with a low relative number of
AET-SRBC-rosetting cells and a high relative number of SIg+ cells (Table 1). Special studies revealed that approximately 70% of all lymphoid cells from this patient reacted with specific anti-μ and anti-κ sera. The absolute number of AET-SRBC-rosetting cells was normal, but the absolute number of SIg+ cells was high (Fig. 1). The labeling index of SIg− blood lymphoid cells was higher than that of corresponding SIg+ cells (Table 1). This observation was confirmed by cytophotometric DNA measurements (Table 2). Labeled cells (SIg− and SIg+) were leukemic blasts. This patient died in relapse 7 months after diagnosis.

The last patient (M.N.), considered to have T-ALL, was admitted because of a large mediastinal mass and respiratory distress. She had a high initial WBC, a low relative number of SIg+, and a very high relative number of AET-SRBC-rosetting cells (Table 1). The absolute number of AET-SRBC-rosetting cells was 16 times the upper limit of the normal range, and also, the absolute number of SIg+ cells was greater than normal (Fig. 1). Both the labeling index determinations (Table 1) and the cytophotometric DNA measurements (Table 2) indicated that the SIg−, but not the SIg+, cells proliferated. Practically all labeled cells were leukemic blasts. This patient died in relapse 10 mo after diagnosis.

**DISCUSSION**

Possible pitfalls inherent in methods for the detection of SIg and AET-SRBC-rosetting cells have been discussed extensively elsewhere. For sequential SIg and cytokinetic studies, our F (ab')2 antisera were found to give less intensive fluorescence than the intact [non-F(ab')2] antisera used for this study. In order to exclude monocytoid cells with Fc receptors, the morphology of mapped cells was carefully evaluated by staining the smears after autoradiography. Despite the preceding multistep processing, the morphology of the mapped cells was well preserved. The number of monocytoid cells excluded varied from patient to patient, but was usually small due to the fact that cells with monocytoid features were already differentiated from lymphoid cells during the mapping procedure. Unspecific binding of the antisera by Fc receptors of the reaminging lymphoid cells was small, as assessed by the difference in the relative number of SIg+ cells after use of non-F(ab')2 and F(ab')2 sheep antisera.

Assumptions made in the interpretation of autoradiographic results based on the use of 3HdT have also been previously discussed. It was of interest to note that, in this study, there was a good correlation between the labeling index data and the cytophotometric determinations of nuclear DNA content.

For an optimum characterization of leukemic cells, marker studies are best performed on bone marrow samples if adequate numbers of cells are available. For investigations of possible interactions between leukemic and normal lymphoid cells or for studies of extramedullary differentiation processes, the assessment of lymphoid cells in peripheral blood by sequential immunologic and cytokinetic methods may be more rewarding.

Our cytokinetic studies on SIg+ and SIg− lymphoid blood cells in children with non-T−non-B ALL revealed that in all patients, labeled SIg− cells were found but that only in 4 of 7 were labeled SIg+ cells present. Morphologically, practically all labeled cells appeared as blast cells. There appeared to be no correlation between the initial white blood cell count and the presence or absence of labeled SIg+ cells. The labeling index of SIg− cells was always higher than that of SIg+ cells. In the bone marrow, no proliferating SIg+ cells were found in one of three children investigated. In one child, labeled SIg+ cells were found in the bone marrow but not in the blood.

These observations are consistent with the findings of others, indicating heterogeneity of non-T−non-B ALL. They suggest that ALL cells differentiate to varying degrees and that proliferative activity decreases with increasing differentiation.

One could argue that the fluorescence of labeled SIg+ cells was unspecific. If this were true, it would be astonishing to find that, in patient T.B., none of 488 SIg+ but 8 of 429 SIg− cells had a hyperdiploid nuclear DNA content (Table 2). Since, in patients T.B. and C.J., the results of the autoradiographic and cytophotometric studies (Tables 1 and 2) correlated well, the existence of nonproliferating lymphoid blood cells with hyperdiploid nuclear DNA content appeared unlikely.

It is of interest to note that in three of the patients with a low initial white blood cell count, the absolute number of SIg+ and/or AET-SRBC-rosetting blood cells was reduced (Fig. 1), despite the fact that proliferating cells were circulating. Since, at least morphologically, the majority of unlabeled lymphoid cells appeared to be normal lymphocytes, the defective production of red cells, granulocytes, and platelets may have been associated with a derangement of an early lymphocyte precursor. With regard to patients with non-T−non-B ALL characterized by a high initial white count, it would be important to know whether the absolute number of labeled SIg− and/or SIg+ lymphoid blood cells is of prognostic significance.

Patient F.J. had a leukemic monoclonal B-cell disease, similar to that described by Flandrin et al. The sequential surface marker and cytokinetic studies on individual lymphoid blood cells revealed that, in this patient also, the labeling index of SIg− cells was higher than that of SIg+ cells, indicating different
degrees of differentiation among the leukemic cells. In addition, the cytophotometric DNA measurements demonstrated that there was no marked loss of SIg in a given phase of the cell cycle. Patient M.N. had T-cell ALL. From the data in Table 1 and Figs. 1 and 2, it appears that AET-SRBC-rosetting blood cells must have been labeled. Despite the fact that in patient M.N. the absolute number of SIg+ blood cells was above the normal range, neither a single labeled SIg+ cell (Table 1) nor a single SIg+ cell with a hyperdiploid nuclear DNA content was found.

In conclusion, the preliminary results of our investigations suggest that sequential immunologic and cyto-
kine studies on individual leukemic cells may permit the distinction of non-T--non-B ALL with from those without proliferating SIg+ lymphoid cells in the blood. It appears that the presence or absence of proliferating SIg+ cells is not related to the initial white blood cell count, but further studies are requi-
ted to confirm this point. Our studies also show that there was no important loss of SIg during the S and G2 phases of the cell cycle.

ACKNOWLEDGMENT

We are grateful to A. Steiner for expert technical help. We thank Dr. F. Skvaril for generously providing the antisera.

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