Shifts in Expression of Cell Membrane Phenotypes in Childhood Lymphoid Malignancies at Relapse

By L. Borella, J. T. Casper, and S. J. Lauer

To determine if cell membrane phenotypes change under the selective pressures of therapy we have conducted a prospective study of 54 children with lymphoid malignancies of T-like, B-like, common, and null cell types. Membrane phenotypes were determined at diagnosis in all patients and again 1–24 mo later in 18 children who either failed induction therapy or had one or more relapses. In 7 patients the cells tested were from relapse sites different than those of the original diagnoses. The data indicate that at relapse most children with lymphoid neoplasias had the same cell membrane phenotype as established at diagnosis, and suggest that the site of relapse did not affect the expression of cell surface markers. However, there were three exceptions: (1) a child initially diagnosed as having null cell acute lymphocytic leukemia had 90% T-antigen-positive blasts in her second-relapse bone marrow; (2) only membrane IgM was present on relapse blasts from a B-cell lymphoma that had both membrane IgM and IgD before initiation of treatment; (3) at diagnosis, bone marrow blasts from a child with T-like leukemia expressed both T antigen and E receptors, but at relapse, bone marrow and pleural fluid cells expressed only T antigens. We postulate that these phenotype shifts may be due to selective effects of therapy on cells at different stages of differentiation.

In spite of major advances in the treatment of leukemia, about one-half of children with lymphoid malignancies still die from their disease. The major obstacle to cure of leukemia in these patients is initial or acquired resistance to antineoplastic agents. The mechanisms of drug resistance and the pathogenesis of leukemia relapse are still poorly understood. It is not known whether the selective pressures of therapy induces drug resistance in an originally sensitive cell population or allows the growth of an initially resistant leukemic cell clone. A first step in answering this question would be to compare the biologic and pharmacologic characteristics of leukemic cells at diagnosis with those of cells obtained when the tumor recurs.

The aim of this study was to determine if leukemic blasts express the same membrane phenotype at diagnosis and relapse. Although it is now accepted that the presence of membrane differentiation markers at diagnosis distinguishes various subtypes of childhood acute lymphocytic leukemia (ALL) and lymphoma, it is uncertain if these membrane phenotypes persist or change under the selective pressures of therapy. Therefore, we conducted a prospective study of 54 children with lymphoid malignancies of T-like, B-like, common, and null cell types.
Membrane phenotypes of lymphoid neoplastic cells were determined at diagnosis in all patients and again 1–24 mo later in 18 children who either failed induction therapy or experienced one or more relapses during the continuation phase.

**MATERIALS AND METHODS**

**Cell Preparation**

Cells were obtained from hepaninized bone marrow aspirates, pleural fluid, cerebrospinal fluid (CSF), or lymph node biopsy performed for diagnostic purposes in 49 children in ALL and 5 children with non-Hodgkin's lymphoma. Ages ranged from 4 mo to 16 yr. All samples contained 70%–99% lymphoid cells.

Red cells were removed from the cell-rich plasma, bone marrow, and pleural fluid specimens by sedimentation at 37°C. CSF samples were centrifuged, and the cell pellet was washed two times in phosphate-buffered saline (PBS), pH 7.4. Biopsy specimens were minced into small fragments, and cells were teased out by passing the free cells through a 2-ml syringe with needles of increasing gauge. All cell preparations were washed with Hanks' balanced salt solution and counted, and viability was determined using trypan blue.

**Detection of Membrane Immunoglobulin and E-Rosette Formation**

Membrane Ig (mlg) was determined by direct immunofluorescence of viable cells incubated at 4°C for 30 min with fluorescein-conjugated goat antihuman Ig (Meloy). Samples that demonstrated mlg were further tested for the presence of γ, α, µ, ε, and δ heavy chains and κ and λ light chains using monospecific goat antisera. The proportions of E-rosette-forming cells were established using methods previously described. Rosettes formed at 4°C by normal lymphocytes dissociate at 37°C, whereas high proportions of thymocytes and E-rosette ALL blasts form rosettes that are stable at 37°C. Therefore, the numbers of rosettes were determined in replicate samples previously incubated at 4°C and 37°C for 1 hr.

**Preparation, Absorption, and Characterization of Heterologous Antisera to Human Thymus and Non-T Non-B ALL Blasts**

Rabbits were inoculated with cells obtained from normal human thymus (anti-T serum) or with ALL lymphoblasts that did not express mlg and did not form E rosettes (anti-ALL serum). Cell doses, schedules, routes of immunization, absorption procedures, and reactivity have been described in detail. By immunofluorescence, anti-T serum reacted with 72%–99% of human thymocytes, 28%–58% of blood lymphocytes, and 1%–8% of bone marrow lymphocytes. This antiserum also reacted with ALL blasts that formed E rosettes. Anti-ALL serum reacted with antigens expressed by ALL blasts that did not form E rosettes and did not express T antigens. This antiserum did not bind to thymocytes, blood lymphocytes, pokeweed-mitogen- and photohemagglutinin-induced blasts, or chronic lymphocytic leukemia (CLL) cells bearing mlg.

**Detection of Cell Surface Antigens by Immunofluorescence**

Viable lymphoid cells (1.5 x 10⁶) were first incubated for 30 min at 4°C with 50 μl of test serum or normal rabbit serum at dilutions ranging from 1:2 to 1:50. The cells were then washed with PBS, pH 7.4, three times and incubated with 50 μl of fluorescein-conjugated goat antirabbit Ig (Meloy). After 30 min at 4°C, the cells were again washed three times with PBS and viewed using a fluorescence microscope. The results were expressed as percentage positive immunofluorescent cells. All tests included a negative control consisting of normal rabbit serum at the same dilution. The numbers of positive cells with the control serum ranged from 1% to 3%.

In this study we used the same immunologic classification of ALL recently reported by Chessells et al. Patients with lymphoblasts that formed E rosettes and/or expressed T antigens were classified as having T-like leukemia-lymphoma. Those with lymphoblasts bearing mlg were diagnosed as having B-like leukemia-lymphoma. Patients whose lymphoblasts did not form E rosettes, express T antigens, or bear mlg, but reacted with anti-ALL sera, were identified as having common ALL, whereas those with lymphoblasts without any of the preceding markers were considered as having null cell ALL.
EBV studies were performed by Dr. W. Henle, Philadelphia, Pa.

Table 1. Initial Immunologic Diagnoses and Clinical Features in 54 Children With Lymphoid Neoplasias

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>T-like</th>
<th>B-like</th>
<th>Common</th>
<th>Null Cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients</td>
<td>10</td>
<td>5</td>
<td>37</td>
<td>2</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>8/2</td>
<td>5/0</td>
<td>16/21</td>
<td>0/2</td>
</tr>
<tr>
<td>Age</td>
<td>12 yr*</td>
<td>12 yr</td>
<td>5 yr</td>
<td>4 mo, 9 yr</td>
</tr>
<tr>
<td></td>
<td>(2–17 yr)</td>
<td>(10–12 yr)</td>
<td>(6 mo–16 yr)</td>
<td></td>
</tr>
<tr>
<td>WBC† (x 10^3/cu mm)</td>
<td>127</td>
<td>10</td>
<td>7</td>
<td>39, 19</td>
</tr>
<tr>
<td></td>
<td>(3.4–364)</td>
<td>(8.9–13)</td>
<td>(0.6–288)</td>
<td></td>
</tr>
<tr>
<td>Bone marrow involvement at diagnosis</td>
<td>7</td>
<td>3</td>
<td>37</td>
<td>2</td>
</tr>
<tr>
<td>Mediastinal mass</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Median, range in parentheses.
†WBC of patients with bone marrow involvement at diagnosis.

RESULTS

Immunologic Diagnosis by Cell Membrane Phenotype

Cell membrane phenotypes were determined on bone marrow blasts or extra-medullary tumor cells obtained at diagnosis from 54 children with lymphoid malignancies. The clinical data summarized in Table 1 essentially confirm previous reports.2,3,5,7 Blasts from 10 patients expressed T antigen (s), and they formed E rosettes in 9 of the 10 patients. T-like disease was more common in older boys and was frequently associated with high WBC counts and anterior mediastinal masses. The child whose blasts were T-antigen-positive and E-rosette-negative was a 2-yr-old girl with a high WBC count but no thymic mass.

B-like disease was diagnosed in 5 prepubertal boys. Although 3 of the 5 boys had bone marrow involvement at diagnosis, their peripheral WBC and differential counts were within normal limits. These 5 patients presented with adenopathy and abdominal tumors associated with ascitic and/or pleural fluid. Their lymphoblasts expressed mIgM, which in 3 patients was also associated with IgD. The light chains were λ in 4 patients and κ in 1 patient. Nuclear antigen associated with Epstein-Barr virus (EBV) was not detectable in the tumor cells, and the patients’ sera had no antibodies to EBV-induced early antigens.*

Bone marrow blasts from 37 children neither formed E rosettes nor expressed T antigen(s) or mIg, but they reacted with heterologous antiserum to non-T non-B ALL blasts (ALL antiserum). The median age of these patients was 5 yr, and the male-to-female ratio was 0.8. Two-thirds of them had WBC counts lower than 15,000/mm³ and none presented with mediastinal enlargement.

Two children could not be assigned to one of the three groups previously mentioned. The lymphoblasts from a 4-mo-old girl did not react with any of the antisera and did not form E rosettes. The bone marrow from a 9-yr-old girl contained 56% blasts and 42% small lymphocytes, which were normal by cytomorphologic criteria. Thirty-eight percent of the bone marrow cells formed E rosettes. However, the rosette-forming cells were small lymphocytes, and the rosettes dissociated after 1 hr of incubation at 37°C. Low proportions of these small cells reacted with anti-T (13%) and anti-ALL (6%) sera. Since their lymphoblasts did

*EBV studies were performed by Dr. W. Henle, Philadelphia, Pa.
not express any of the markers tested, these 2 patients were classified at diagnosis as having null cell ALL.\textsuperscript{7}

**Lymphoblast Cell Membrane Phenotype Following Induction Failure or Relapse**

Eighteen of these 54 children either failed to achieve remission (1 of 18) or relapsed one or more times (17 of 18), thus providing the opportunity to retest membrane phenotypes of leukemic cells that had been exposed to antileukemic drugs.

**Immunologic subtype at diagnosis and relapse.** Thirty samples were obtained from these 18 children at intervals ranging from 1 to 20 mo after diagnosis. The initial phenotype was T-like in 3, B-like in 3, common type in 11, and null cell type in 1. The only immunologic subtype change was detected in a 9-yr-old girl who had initially been classified as having null cell ALL. Her phenotype shifted to T-like ALL in her second bone marrow relapse (patient R.M., Table 2).

**Alteration of cell surface markers: proportion of positive cells and immunofluorescent pattern.** Table 2 presents the data on samples obtained from the same physical compartment, e.g., bone marrow or pleural cavity, in 14 children who experienced one or more relapses. With one exception (patient A.B.), the percentages of cells reactive with ALL antiserum in children with common ALL (10 patients) were similar or lower in the relapse marrow than in the original sample. Four patients (K.L., F.M., J.Q., L.S.) had 40%-65% reductions in the proportions of cells positive with this antiserum at the time of relapse. Whereas the initial specimens from these 4 patients had only 3%-5% cells bearing T antigen(s) and mIg, the percentages of lymphoid cells bearing T and B markers in the relapse bone

<table>
<thead>
<tr>
<th>Cell Membrane Phenotype</th>
<th>Patients</th>
<th>Diagnosis</th>
<th>1st Relapse</th>
<th>2nd Relapse</th>
<th>3rd Relapse</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ALL T mlg</td>
<td>ALL T mlg</td>
<td>ALL T mlg</td>
<td>ALL T mlg</td>
</tr>
<tr>
<td>A.B.</td>
<td>30</td>
<td>2 1</td>
<td>83 3 0</td>
<td>68 4 0</td>
<td></td>
</tr>
<tr>
<td>T.J.</td>
<td>75</td>
<td>3 0</td>
<td>49 0 5</td>
<td>71 1 3</td>
<td>76 4 3</td>
</tr>
<tr>
<td>K.L.</td>
<td>74</td>
<td>ND$</td>
<td>38 10$ 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F.M.</td>
<td>70</td>
<td>5 0</td>
<td>32 10$ 0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>J.Q.</td>
<td>82</td>
<td>3 0</td>
<td>50 23$ 7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L.S.</td>
<td>55</td>
<td>5 0</td>
<td>19 8 6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>J.B.</td>
<td>51</td>
<td>5 0</td>
<td>34 ND ND 35</td>
<td>3 0</td>
<td></td>
</tr>
<tr>
<td>C.J.</td>
<td>39</td>
<td>4 1</td>
<td>37 2 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S.T.</td>
<td>70</td>
<td>2 0</td>
<td>52 0 0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A.Z.</td>
<td>62</td>
<td>2 0</td>
<td>65 6 0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P.K.</td>
<td>1</td>
<td>82 0</td>
<td>6 84 ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B.N.</td>
<td>4</td>
<td>65 0</td>
<td>3 63 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T.D.</td>
<td>7</td>
<td>70 0</td>
<td>0 98 0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Null</td>
<td>R.M.</td>
<td>6 13$ 1</td>
<td>10 3 0 7 90</td>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>

*Cells were from bone marrow in all patients, except T.D. Diagnosis and relapse lymphoblasts in T.D. were from pleural fluid.*

†ALL: ALL antigens; T: T antigens; mIg: membrane immunoglobulin.

‡By phase microscopy these cells appeared to be small lymphocytes.

§ND: not done; samples did not contain enough cells to perform all tests.
marrows ranged from 10% to 30%. These cells were identified as small lymphocytes by phase microscopy. Myeloid and erythroid cells were present in large numbers in these specimens as compared with the original marrows. We were unable to detect any difference in fluorescent pattern or intensity between cells obtained at diagnosis and relapse.

The most striking change in the expression of membrane phenotype was on blasts from a child initially diagnosed as having null cell ALL (patient R. M., Table 2). Her proportions of T-antigen-positive bone marrow cells were low at diagnosis and first relapse but rose to 90% in her second bone marrow relapse. The percentages of mlg- and ALL-antigen-positive cells remained low and did not change significantly from those of the initial specimen. Although at the time of her second relapse 90% of marrow cells were blasts and were positive with anti-T serum, they never formed E rosettes throughout the course of her disease.

Site of relapse and phenotypic expression. Seven patients relapsed in different sites (Table 3). Two of these had common-type ALL, 2 T-like ALL, and 3 B-cell neoplasia. The two children with common-type ALL developed CNS leukemia. Prior to this episode, one of them (D.K.) had a testicular relapse. Lymphoblasts from the spinal fluids and testicular biopsy did not have T- or B-cell markers, but expressed ALL antigen, as did the original diagnostic bone marrows. Thus the site of relapse did not affect the expression of this membrane antigen. Two children with T-like ALL who presented with mediastinal masses had 63%–84% T-antigen-positive cells in their diagnostic and first-relapse marrows. In each case recurrence of the mediastinal tumor was associated with a lymphoblastic pleural effusion. Cells obtained by thoracentesis were positive with T antiserum and did not express mlg or ALL antigen. However, in patient B.N. the T-positive lymphoblasts obtained from the relapse bone marrow and the pleural fluid did not form E rosettes. The loss of E receptors on lymphoblasts obtained from two different

<table>
<thead>
<tr>
<th>Table 3. Membrane Phenotypes on Lymphoblasts Obtained From Different Sites at Diagnosis and Relapse</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Patient</strong></td>
</tr>
<tr>
<td>T.J.</td>
</tr>
<tr>
<td>D.K.</td>
</tr>
<tr>
<td>P.K.</td>
</tr>
<tr>
<td>B.N.</td>
</tr>
<tr>
<td>B.G.</td>
</tr>
<tr>
<td>J.F.</td>
</tr>
<tr>
<td>S.R.</td>
</tr>
</tbody>
</table>

* Patients T.J., P.K., and B.N. also relapsed in the same site as the original diagnosis site and are included in Table 2.
† BM: bone marrow; LN: lymph node; AT: abdominal tumor; AF: ascitic fluid; CSF: cerebrospinal fluid; T: testicle; PF: pleural fluid.
‡ Numbers in parentheses are percentages of positive cells.
Relapse sites indicated that this was a generalized phenomenon rather than one related to local environmental influences.

Blasts from 3 children with B-cell leukemia-lymphoma obtained from different sites at diagnosis and relapse possessed monoclonal mlg. At diagnosis, 2 patients had mlgM and mlgD on high proportions of blasts. At relapse, however, mlgD was no longer demonstrable on the cells from 1 child and was present on a lower proportion of blasts from the other. Since we were unable to retest cells obtained from the same site, it is uncertain whether or not the disappearance of IgD-bearing cells at relapse was related to the different relapse site.

DISCUSSION

This study demonstrates that at relapse the majority of children with lymphoid neoplasias express the same cell membrane phenotype as established at diagnosis. This is supported by previous studies in smaller numbers of patients. Tsukimoto et al. retested 5 children with ALL and demonstrated the persistence of E rosettes on blasts obtained at diagnosis and relapse from 2 patients with T-cell ALL. The lymphoblasts from the other 3 patients did not express mlg or E receptors at presentation or bone marrow relapse. However, these cells were not tested for the presence of T antigens or ALL antigens. Roberts et al. retested 18 patients at relapse and found that 11 retained the same phenotype detected at diagnosis. This group included children and adults with ALL and CML in blast crisis. Of the 7 patients who had phenotype shifts, 4 had ALL. In a more recent study of 9 children with ALL who were retested at relapse, there was 1 who, in similarity to R.M. (Table 2), had phenotype shifts from T-antigen-negative E-rosette-negative at diagnosis to T-antigen-positive E-rosette-negative at relapse. In contrast to our patient, this child also developed a mediastinal mass at the time of bone marrow relapse.

This study demonstrates that patients with common-type ALL may have significantly lower proportions of cells with ALL antigens at relapse than in the initial bone marrow. It has previously been shown that immunologic diagnosis of childhood leukemia based solely on peripheral blood cell phenotypes may be misleading because of the high proportion of normal lymphocytes with T and B markers and the low percentage of blasts in the circulating pool. A similar problem was encountered in the evaluation of marrow samples from asymptomatic patients who were found to be in relapse during routine bone marrow examinations. The lower percentages of cells bearing ALL antigens in some relapse bone marrows, as compared with the initial specimens, suggested partial suppression of these leukemia-associated antigens at the time of leukemic recurrence. However, the presence in these bone marrows of high proportions of normal T and B lymphocytes plus other nonlymphoid cells indicated that the lower numbers of ALL-antigen-positive cells were due to dilution of leukemic cells with normal marrow cells. These results again emphasize that valid interpretation of data depends not only on simultaneous determinations of various phenotype markers but also on careful evaluation of cell heterogeneity within the sample.

Although E receptors and T antigens are frequently expressed on the same cell, there is no definitive proof that they are part of the same molecule. The blocking of E-rosette formation by anti-T serum only suggests a close association between E
receptors and T antigens. Moreover, it has been reported that at the time of diagnosis there may be dissociation between E receptors and T antigens on leukemic blasts. At presentation, 1 of our patients had lymphoblasts that expressed T antigens but did not form E rosettes. In addition, another child presented with lymphoblasts that expressed E receptors and T antigen(s) but no longer formed E rosettes when the tumor recurred. This phenomenon may be related to charge and/or a conformational alteration of the cytoplasmic membrane. The selection of a T-antigen-positive E-receptor-negative cell subpopulation by therapy is also a possibility.

Ontogenic studies in humans and experimental animal models have indicated that lymphocytes bearing only mIgM develop earlier than do cells expressing both IgM and IgD. Since identification of one or two heavy chains on normal B cells appears to discriminate between sequential stages of B-lymphocyte differentiation, it is possible that this criterion may also apply to B-cell lymphoma or leukemia. Neoplastic cells at various stages of maturation have been described within single tumors, e.g., neuroblasts and ganglion cells in neuroblastoma. Therefore, a plausible hypothesis is that lymphoblasts bearing only mIgM or expressing both mIgM and mIgD are neoplastic cells at different stages of differentiation. A corollary to this hypothesis is that the disappearance of lymphoblasts bearing both IgM and IgD at relapse (patient J.F., Table 3) may be due to the selective effects of therapy on the more thoroughly differentiated cell population, with concomitant proliferation of the more immature cells (mIgM-bearing blasts). A possible approach to test this hypothesis would be to investigate in vitro the differentiation of B lymphoblasts and determine if cells bearing one or more mIgs differ in sensitivity to various antileukemic agents.

Recognition of changes on the neoplastic cell surface at the time of leukemic relapse may increase our understanding of the pathogenesis of tumor recurrence and of the selective effects of therapy on cell subpopulations. This study represents an initial step toward this goal. Future research should include sequential assessment of additional immunologic markers (such as C3 and Fc receptors, mIg idiotypes, and cytoplasmic Ig) and a prospective analysis of cell-associated enzymes, chromosome markers, and pharmacologic phenotypes. By the use of such methods it should be possible to determine if antineoplastic agents selectively suppress tumor cells at various stages of differentiation.

The results of this study may be of immediate practical relevance. Persistence of antigenic molecules on the cell surface at relapse could provide potential immunologic targets at a time when these cells are drug-resistant. Although the antigens identified in this study are not leukemia-specific, they should not be excluded as candidates for new immunotherapeutic approaches. This possibility is supported by recent reports describing regression of a T-cell lymphoma following administration of antithymocyte globulin and the potential use of antisera against mIg idiotypes expressed on B lymphoblasts.

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REFERENCES


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