Immunological Definition of Leukemic Cell Surface Phenotypes

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Abstract

Immunological phenotyping of blasts from over 200 children with acute lymphocytic leukemia (ALL) reveals both interpatient differences and phenotypic heterogeneity in the blast population from individual patients. A battery of five independent lymphocyte differentiation markers, erythrocyte-forming rosettes, T-cell antigens, la-like antigens, the common ALL antigen, and surface immunoglobulin, permit classification of all ALL specimens into four major marker groups. These are common, T-cell, B-cell, and undifferentiated ALL. Heterogeneity in the marker phenotypes within each of the major groups is observed. Within individual erythrocyte receptor-positive ALL specimens, phenotypic heterogeneity in the blast population is demonstrated. Sequential determinations of the blast phenotype during periods of active disease reveal a second example of intrapatient blast cell heterogeneity. Differences in phenotype of the dominant blast populations present prior to treatment and at relapse are observed in sequential studies of individual patients. These shifts in phenotype are nonrandom. They result most frequently from losses in single differentiation markers. A unifying hypothesis which explains these observations of phenotypic heterogeneity is that ALL blasts manifest limited lymphoid-like differentiation.

Introduction

In ALL, only a small minority of blast specimens can be demonstrated to be positive for E-rosette formation or positive for surface immunoglobulin, the respective hallmarks of differentiated T-cells and B-cells. The vast majority of ALL specimens, although lacking these 2 markers, can be positively identified by other surface markers (1, 3). Firm evidence of commitment to T-cell or B-cell differentiation is, however, generally lacking. The common form of ALL is antigenically similar to some cells in fetal liver and in regenerating bone marrow (4). Therefore, common ALL blasts are generally considered to be arrested in an early state of lymphocyte differentiation. Additional steps in differentiation linking this presumed precursor to more mature T- or B-lymphocytes are assumed to exist. To the extent that ALL blasts are representative of immature lymphoid cells, cell marker studies of these lymphoblasts may lead to the identification of additional early differentiation steps, and/or steps which are restricted in the normal individual to a small number of cells. For determination of ALL blast cell phenotypes, we test for the lymphocyte differentiation markers E-rosette formation, T-cell antigens, la-like antigens, the common ALL antigen, and surface immunoglobulin. Although ALL blasts obviously fail to differentiate to end cells in vivo, the careful examination of fresh leukemia specimens may provide evidence of progression through individual differentiation steps. The data presented here document interpatient as well as intrapatient heterogeneity in blast phenotype. Although alternative explanations can be offered for any of the individual observations, the concept that surface marker heterogeneity is a manifestation of limited lymphoid differentiation in ALL blasts provides a unifying theme.

Materials and Methods

Patient Population. Children with ALL admitted to St. Jude Children's Research Hospital from September 1976 to February 1980 were tested for their blast phenotypes prior to treatment. Sequential marker studies were performed on children who developed recurrent disease during this same time interval. These investigations were conducted with the approval of the St. Jude Clinical Trials Committee.

Cell Preparations. Bone marrow aspirates were drawn in heparin, and the cells were separated on a Ficoll-Hypaque gradient. Cells from the gradient interface were washed 3 times in Medium 199 adjusted to a final concentration of 10^6 cells/ml. A drop of the cell suspension was used to prepare Wright's-stained slides for differential counts.

E-Rosette Tests. Three versions of the E-rosette test were used on every patient sample. Blasts were tested for E-rosette formation with untreated sheep erythrocytes by coincubation for 1 hr at 37° or at 4°, and with sheep erythrocytes treated with AET (2, 5). A parallel control of normal blood lymphocytes was used for every patient sample.

Briefly, 100 µl of the lymphocyte or lymphoblast suspension were mixed with an equal volume of a 0.5% suspension of sheep erythrocytes, incubated at 37° for 5 min, and centrifuged at 200 x g for 5 min. Tubes containing the untreated sheep erythrocytes were examined immediately; tubes containing the untreated sheep erythrocytes were examined after the appropriate incubations. The cell pellets were gently resuspended, and a drop of the cell suspension was placed in a hemocytometer. Four hundred nucleated cells from each tube were examined for E-rosette formation. Only cells with 3 or more attached sheep erythrocytes were counted as forming rosettes. Wright's-stained slides were prepared to determine the morphology of the rosette-forming cells. Patients' samples were considered E-rosette positive only if the rosette-forming cells were lymphoblasts. The percentage of E-rosette-forming blasts in each sample was corrected for the presence of nonlymphoid, nonsetting cells, where necessary.

Antisera. Two rabbit antisera to human T-cells were used. The first was an anti-thymocyte serum prepared as previously described (5). The second was prepared against ALL blasts that were reactive with the first serum. This second T-cell serum was absorbed in the same manner, except that it required additional absorptions with the common ALL-like cell line, NALM-1. Both sera were reactive with blood T-cells, thymocytes, E-rosette-positive ALLs, and a minority of E-negative ALLs. The anti-la-like serum was obtained commercially (Alpha Gamma Laboratories, Sierra Madre, Calif.). The antisemur to the common ALL antigen has been extensively characterized (6). It was prepared by immunization of a rabbit with ALL blasts which lacked both T-cell markers and surface immunoglobulin. The serum was rendered specific for the common ALL antigen by screening with 100 µl of NALM-1 cells, followed by one additional absorption with NALM-1 cells.

Determination of la-like Antigen Content. The la-like antigen content of blasts was determined by a modification of the method of Belknap (7). The la-like antigen has been extensively characterized (8). It was prepared by immunization of a rabbit with ALL blasts which lacked both T-cell markers and surface immunoglobulin. The serum was rendered specific for the common ALL antigen by screening with 100 µl of NALM-1 cells, followed by one additional absorption with NALM-1 cells.

Determination of E-rosette Activity. E-rosette formation was determined by the method of Imrie et al. (9). E-rosette-forming blasts in each sample were corrected for the presence of nonlymphoid, nonsetting cells, where necessary.

References

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2. The abbreviations used are: ALL, acute lymphocytic leukemia; E-rosette, erythrocyte-forming rosette; AET, 5-(2-aminoethyl)isothiouronium bromide hydrobromide.
for non-T, non-B ALL blasts by exhaustive absorption with human RBC
 tonsil cells, and bone marrow cells.

**Immunofluorescence.** Direct immunofluorescence with a polyvalent
gost anti-human immunoglobulin (Meloy Laboratories, Inc., Springfield,
Va.) was used to determine the presence or absence of surface
immunoglobulin. Indirect immunofluorescence was used to determine
the presence or absence of the other surface antigens (5, 8). Briefly,
aliquots (100 µl) of the test cell suspension were washed in cold 0.01M
phosphate-buffered saline containing 5% γ-globulin-free calf serum
and 0.08% sodium azide. The packed washed cells were incubated
with 50 µl of the appropriate rabbit serum for 30 min at 4°. The cells
were washed 3 times, followed by incubation with fluorescent goat
anti-rabbit immunoglobulin (C alfiochem-Behring Corp., La Jolla, Calif.)
for 30 min at 4° and 3 additional washes. The cells were resuspended
in 60% glycerol in phosphate-buffered saline, and slides were
prepared. They were coded, and the percentage of cells with positive
surface fluorescence was determined by 2 trained observers. Blast
specimens which contained 45% or more positive cells were consid-
ered positive for the marker being tested (8).

**Results and Discussion**

Four separate observations demonstrate phenotypic hetero-
genicity of blasts from children with ALL: (a) different patients have blasts which bear different patterns of lymphocyte differ-
entiation markers; (b) at least some of these phenotypes can
be related to each other by single, stepwise changes in individ-
ual surface markers; (c) similar phenotypic variants and relat-
edness of phenotype are observable within the blast population
from individual patients; (d) frequent shifts in the phenotype of
the dominant blast population of individual patients are ob-
erved from the pretreatment phenotype to a nonidentical but
related phenotype at relapse. These evidences of related phe-
notypes in ALL suggest a stepwise sequence possibly related to
or analogous to normal lymphoid differentiation.

Using 5 independent lymphocyte differentiation markers, E-
orosette formation, T-cell antigens, la-like antigens, the common
ALL antigen, and surface immunoglobulin, 4 major groups of
ALL can be identified. Table 1 illustrates the interpatient differ-
ences in marker patterns and the frequency of the major
phenotypic groups at diagnosis for previously untreated chil-
dren with ALL. The group assignments are based on the pattern
of markers expressed, rather than on the presence or absence
of a single marker. Furthermore, these groups are defined as
exclusive groups by assigning some patterns more important
than others. The 4 groups are designated as common, T-cell,
B-cell, and undifferentiated ALL, following the general scheme
introduced by Greaves et al. (4). The major positive markers of
each group are denoted by boxes in the table. Specifically, we
classify patients into the major ALL groups according to the
following surface marker patterns: (a) common ALL, presence
of both la-like antigens and common ALL antigen; (b) T-cell,
presence of one or both T-cell characteristics (E-rosette-posi-
tive and T-cell antigens) and absence of the common ALL
pattern; (c) B-cell, presence of la-like antigens and surface
immunoglobulin; and (d) undifferentiated ALL, absence of T-
cell, B-cell, and common ALL patterns. The undifferentiated
group includes samples which bear either the la-like antigens
or no detectable surface markers. The group frequencies given
are based on the blast phenotypes of 182 of 200 consecutive
children with ALL admitted to our institution. The 18 exclusions
are children whose marrow specimens had inadequate num-
bbers of blasts. The largest group of patients is the common ALL
group, which includes 69% of our patients. The T-cell and
undifferentiated groups are smaller, represented by 18 and
11% of our patients, respectively. The B-cell group is the
smallest, with only 2% of new patients showing this blast cell
pattern.

In an orderly sequence of differentiation, small, perhaps,
single, changes in surface markers are expected to distinguish
individual transitional states of differentiation. The second link
between blast phenotype and differentiation is the observation
that blasts from some patients have marker patterns which
are intermediate to those marker patterns which distinguish the
more frequent, major phenotypic groups. These blasts may be
arrested in intermediate and previously unrecognized differ-
tentiation steps. As illustrated in Table 1, there are some
markers which may or may not be present on blasts character-
ized as belonging to the same ALL group.

For instance, reproducible phenotypic heterogeneity in T-
cell ALL is demonstrable by this battery of standard surface
markers. Approximately 9% of the children with ALL have
blasts that form E-rosettes in all 3 assays. An additional 5% of
patients have blasts which form rosettes only with the AET-
treated erythrocytes (5). Most of these additional samples can
be identified as T-cell ALL by independent criteria. These are
the presence of T-cell antigens and the fact that many of these
patients have the clinical features usually associated with E-
orosette-positive or T-cell ALL (7). These observations, coupled
with the fact that the phenotype is repeatedly observed, leads
us to conclude that this phenotype represents an intermediate
state of differentiation in the T-cell lineage. A reasonable se-
quency of differentiation is that T-antigen-positive cells acquire
the ability to form rosettes with AET-treated erythrocytes as an
intermediate step in maturing to cells capable of forming heat-
stable rosettes. Since normal cells which form rosettes at 37°
are found predominantly in the thymus, the postulated differ-
entiation event must occur either in a subpopulation of early
thymocytes or in an as yet unidentified extrathymic or pre-
thetic cell. Further verification of this postulated intermediate
in T-cell differentiation requires the identification and charac-
terization of this putative normal counterpart.

Several exceptional patients were also identified as having
blasts which formed rosettes only with the treated sheep cells,
but the other surface markers of these blasts classified them
as either undifferentiated ALL or common ALL. Two of these
exceptional patients have since relapsed. We were able to

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**Table 1**

<table>
<thead>
<tr>
<th>Membrane marker</th>
<th>Common</th>
<th>T-cell</th>
<th>B-cell</th>
<th>Undiffer-entiated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythrocyte receptors</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>T-cell antigens</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>Common ALL antigen</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>±</td>
</tr>
<tr>
<td>la-like antigens</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>±</td>
</tr>
<tr>
<td>Immunoglobulin</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

* = marker absent from all specimens; ±, marker may be either present or
absent; +, marker present on all specimens.

Based on 182 untreated children with ALL.
obtain an adequate blast sample at relapse from one patient, and we have confirmed the original unusual blast phenotype. Since these patients are dissimilar and we have no independent means of verifying these phenotypes, the most prudent interpretation at this time must be that rosette formation with AET-treated erythrocytes, while very sensitive for the detection of T-cell ALL, is not entirely specific for this group of ALLs. The rest of the T-cell ALL specimens are E-rosette-negative by all 3 versions of the E-rosette test. They are T-cell antigen positive and lack one or both markers characteristic of common ALL. Of all the T-cell ALLs, approximately 20% also express the common ALL antigen. This wide range of phenotypes within the T-cell ALL group may be indicative of additional individual steps in differentiation which relate one phenotypic pattern to another and one phenotypic group to another.

Although the differentiation state and/or potential of the leukemia stem cell is unknown, a third piece of evidence consistent with differentiation models is the presence of a subpopulation of phenotypically variant blasts within the leukemic cell population of a single individual. These variant phenotypes may be the products of mutant cells or evidence of limited differentiation within the leukemic cell population. There are often blasts within a sample which appear to be negative for a given surface marker. The absence of a surface marker on a subpopulation of blasts can be confirmed if these blasts remain negative when an assay of greater sensitivity is applied. The rosette assays are technically good examples of this point, because the morphology of the individual positive cells confirms the presence of blasts and the discrepancy between total blasts and marker-positive blasts. Chart 1 shows the results obtained with pretreatment ALL samples from 24 different patients that were determined to have some proportion of blasts forming rosettes. The lines connect values obtained on individual specimens at 37°, at 4°, and with AET-treated erythrocytes. The proportion of blasts forming rosettes at 37° ranges from 4 to 71% for these individual patients. Since our mean percentage of normal blood lymphocytes which form rosettes under these conditions is 8%, these E-rosette-positive blasts are more easily detected than are normal blood T-cells. The assay at 4° increases the number of rosette-forming blood lymphocytes by an average of 5-fold; however, with leukemia cells the proportion of cells which form rosettes is generally the same at the 2 temperatures (2, 5). The third assay using AET-treated erythrocytes has been used for the enumeration of total T-cells in normal blood. In our experience, a mean of 81% of normal blood lymphocytes form rosettes under these conditions. The remaining negative cells in normal blood can be accounted for as surface immunoglobulin-positive B-cells (15%) and null lymphocytes (4%). If this means of detecting total T-cells is applied to our E-positive leukemia specimens, we observe an average increase of 2-fold in the proportion of rosette-forming cells. However, the proportion of lymphoblasts forming rosettes in these specimens falls short of the 100% value anticipated in a phenotypically homogeneous population tested with an optimally sensitive detection method. The mean proportion of rosette-forming lymphoblasts is only 74%, and the range is 14 to 94%. These data indicate that within the blast cell population from individual E-positive ALL patients there are blasts which lack the ability to form rosettes even under the most sensitive test conditions. This heterogeneity is not an artifact of suboptimal testing procedures, but a property of the blast cell population itself.

These examples show that we can observe heterogeneity in the blast population from a single patient at a single time point, although this heterogeneity is obviously more difficult to document then differences between samples from different patients. The cause of this heterogeneity is unknown at the present time. Separation and further characterization of positive and negative subpopulations are required to determine how these subpopulations are related to each other. Such heterogeneity may be unimportant in ascribing a phenotype and in determining the cell lineage if, as assumed from a differentiation model, these cells are to some extent interconvertable. The variant blasts may be detectable in the pretreatment specimens or, if not detectable, they may appear at relapse as a phenotypic shift in the blast population. The occurrence of phenotypic shifts is the fourth demonstration of phenotypic heterogeneity in ALL blasts.

These phenotypically variant cells may have clinical importance if this subpopulation has a different growth potential than the majority blast population. If a minor blast population enjoys a growth advantage under the selective pressure of chemotherapy, the initially infrequent or possibly an undetectable phenotype could become the dominant blast phenotype in patients relapsing on therapy. Obviously, the important and immediate test is to determine if the blast cell phenotype at relapse is changed from the phenotype determined prior to treatment and if these phenotypes are related to each other.

With our complete battery of marker tests, we have determined the blast phenotype of 25 patients at least twice during the course of active disease. As with all of our marker tests,

![Chart 1. Heterogeneity of blast population within 24 individual E-rosette-positive ALL specimens. E-rosette formation assayed with untreated erythrocytes at 37°, at 4°, and with AET-treated erythrocytes. Lines connect the percentage of cells forming rosettes in each individual specimen tested under the separate assay conditions.](chart1.jpg)
assay conditions are optimized and standardized to assure reproducibility. Of the 25 patients tested, 12 showed a marker change at a later stage of disease. The individual marker changes observed were nonrandom. Within the T-cell group there were 2 losses of the common ALL antigen. Within the common group there were 3 losses of the same antigen. In the undifferentiated group, by contrast, 3 gains in the common ALL antigen were observed. In our experience, the ALL antigen is the marker exhibiting the most frequent changes. Changes in this antigen have also been reported from Dr. Greaves’ laboratory (6). Other antigen changes we observed were loss of Ia-like antigens (3 patients) and loss of reactivity with one of our T-cell antisera (2 patients). These small, but detectable differences in the dominant blast populations at different stages of the disease suggest that blasts with different phenotypes can be related to each other by a simple, stepwise sequence. In the treated patients these changes may represent mutational, adaptive, or differentiation events that confer a selective advantage on a subpopulation of blasts. The stability of these changes as determined by sequential phenotyping may elucidate the causative events and may suggest the most likely mechanism(s) controlling marker expression. These observations also have important clinical ramifications if a marker like the common ALL antigen is used to eliminate or to measure the absolute number of leukemia cells. Residual blasts lacking the marker could both escape detection and provide the nucleus for the regrowth of the leukemic cell population.

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References