Comparison of Techniques for Detecting T-Cell Acute Lymphocytic Leukemia

By Susan L. Melvin

Three versions of the E-rosette test, one using untreated sheep erythrocytes at 37°C, another using such cells at 4°C, and a third using sheep erythrocytes treated with S-(2-aminoethyl)isothiouronium bromide hydrobromide (AET), were applied to each of 72 bone marrow specimens from as many unselected patients with untreated acute lymphocytic leukemia (ALL). The same specimens were also examined for T-cell antigens, based on reactivity with an antithymocyte serum. Lymphoblasts in eight ALL specimens formed E rosettes at 37°C; no other E-positive specimens were identified when the assay was done at 4°C. With AET-treated erythrocytes, lymphoblasts from these eight specimens and six additional specimens readily formed rosettes. T-cell antigens were detectable in all specimens positive for rosette formation with untreated erythrocytes, in four of the six specimens positive for rosette formation with AET-treated erythrocytes, and in four specimens that showed no rosette formation under any of the experimental conditions used. Altogether, 18 specimens contained lymphoblasts with one or more surface markers characteristic of T-cell leukemia. These findings indicate that more specimens are likely to be identified as T-cell leukemias when E-rosette tests of increasing sensitivity and assays for T-cell antigens are used. Some leukemic blasts do not possess the full array of membrane receptors and antigens usually associated with T cells. A combination of E-rosette tests and serologic tests is necessary to determine reliably the relationship of the test specimen to either T-cell ALL or common ALL and to establish the clinical significance of blasts that express membrane properties intermediate between those of T-cell ALL and common ALL.

Interest in the cell surface features of leukemic lymphoblasts stems from the observation that some blast cells in acute lymphocytic leukemia (ALL) have membrane properties similar to those of normal thymus-derived lymphocytes (T cells). Correlation of these T-cell markers with initial patient features associated with short remissions and with a high frequency of meningeal relapse has established T-cell leukemia as a clinically important form of ALL. Since T-cell ALL is an unusually aggressive disease in many patients, a reliable laboratory basis for recognizing T-cell ALL is essential for the development of improved treatment and for clarification of the biologic bases for these clinical observations. The apparent percentages of ALL patients with T-cell blasts vary from 12% to 35%, and reports of correlations between T-cell markers and individual clinical features at diagnosis are not consistent. These discrepancies could result from differences in the sensitivities of the methods used to detect T-cell properties or differences in the membrane structures being examined.

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DETECTION OF T-CELL LYMPHOCYTIC LEUKEMIA

In the present study, a battery of tests was used to detect T-cell markers on blast cells obtained from a large unselected group of ALL patients at the time of diagnosis. The assays chosen are representative of methods currently being used to type malignant lymphoid diseases. Three versions of the E-rosette test—incubation at 37°C or 4°C and sheep erythrocytes treated with S-(2-aminoethyl)isothiouronium bromide hydrobromide (AET)—were selected because of their differing sensitivities when applied to normal lymphocyte populations. Binding of antithymocyte serum provided an independent means of identifying T cells. Each of these assays has been used to distinguish subsets of normal T cells,10,11 and hence the battery would be expected to provide a more adequate definition of the T-cell features on leukemic blast cells than would any single assay alone.

MATERIALS AND METHODS

Patients

Seventy-two previously untreated children admitted to St. Jude Children's Research Hospital from January 1977 through June 1978 with a diagnosis of ALL were screened for T-cell surface markers on their leukemic blasts. The diagnosis was based on the presence of 25% or more lymphoblasts or undifferentiated blasts in the bone marrow specimen. Included by these criteria were all patients whose blasts did not have unequivocal Auer rods or other evidence of myelocytic, monocytic, or erythroid differentiation. All tests for cell surface markers were done with bone marrow specimens obtained before treatment.

These investigations were conducted with approval of the St. Jude Clinical Trials Committee.

Cell Samples

Two milliliters of heparinized bone marrow were obtained from each patient at the time of diagnosis. Samples were centrifuged, and the plasma was removed. The cells were resuspended in medium 199 (M199) containing 5 U of heparin per milliliter. Erythrocytes were removed, and mononuclear cells were enriched by centrifugation over a Ficoll-Hypaque gradient.12 The mononuclear cells were recovered from the interface, washed three times in M199, and adjusted to a final concentration of 10^7/ml. A drop of the cell suspension was used to prepare Wright-stained slides for differential counts. The median percentage of lymphoid cells in the final cell preparations was 98% (range 27%-100%).

E-Rosette Tests

Fresh pooled sheep erythrocytes in Alsever's solution were obtained weekly (Colorado Serum Company). Each lot was tested for E-rosette formation with normal blood lymphocytes before use with patients' samples. Washed sheep erythrocytes, either untreated or treated with AET for 20 min at 37°C,13 were used in rosette assays as a 0.5% suspension in M199 containing 10% calf serum. The calf serum had been absorbed with sheep erythrocytes and tested in rosette assays with normal blood lymphocytes. The suspensions of untreated erythrocytes were used within 1 day of preparation, whereas sheep erythrocytes treated with AET were stored at 4°C and used within 1 wk of preparation.

An aliquot (100 μl) of the lymphoid cell suspension was mixed with an equal volume of each erythrocyte suspension, incubated at 37°C for 5 min, and centrifuged at 200 g for 5 min. Tubes that contained AET-treated erythrocytes were examined without further incubation; those with untreated sheep erythrocytes were incubated at either 4°C or 37°C for 1 hr.10 The cell pellets were gently resuspended with a Pasteur pipette, and a drop of each of the suspensions was placed in a hemocytometer. Four hundred nucleated cells from each tube were assessed, and the results were expressed as the percentage of cells that formed rosettes. In this assessment the criterion for rosette formation was attachment of three or more sheep erythrocytes to a nucleated cell. Wright-stained slides were prepared if more than 2% of the cells formed rosettes with untreated sheep erythrocytes at 37°C or if more than 5% formed rosettes under the alternate conditions. The fixed slides were used to determine the morphology of the cells that formed rosettes. Patients' samples were considered E-positive only if the rosette-forming cells could be identified as lymphoblasts.
**Preparation of Antithymocyte Serum**

An unabsorbed anti-human-fetal-thymocyte serum derived from a hyperimmune rabbit was provided by Dr. Ronald Acton, University of Alabama, Birmingham, Alabama. The serum was heated at 56°C for 30 min and absorbed with human red cells and Raji cells, a B-cell lymphoblastoid line.\(^1\) Cells for absorption were washed three times in phosphate-buffered saline (PBS), and one volume of the packed cells was mixed with three to five volumes of serum and incubated at 4°C for 1 hr. Absorptions were repeated until the serum showed no further hemagglutinating activity against human red cells that represented the major blood groups and no binding to Raji cells as determined by indirect immunofluorescence. This serum bound to 60% (mean) of blood lymphocytes from normal adults and reacted with the immunoglobulin-negative cells in both blood and tonsil cell preparations.

**Indirect Immunofluorescence Test**

The binding of the absorbed antithymocyte serum in indirect immunofluorescence tests was used to enumerate T cells in the bone marrow specimens from patients with leukemia. Aliquots (100 μl) of separated lymphoid cells were washed three additional times in cold PBS containing 5% gamma-globulin-free calf serum and 0.8% sodium azide. The packed washed cells were resuspended in 50μl of antithymocyte serum, unabsorbed antilymphocyte serum, or normal rabbit serum and incubated at 4°C for 30 min.\(^1\) The cells were washed three times and then incubated at 4°C for 30 min with a fluorescent anti-rabbit-immunoglobulin serum from goats (Behring Diagnostics). After the cells were washed three additional times and resuspended in 60% glycerol in PBS, slides were prepared. They were coded and then interpreted by two trained observers. At least 200 cells were assessed, and the results were expressed as the average percentages of cells with membrane fluorescence. The background of positive fluorescent cells obtained after incubation with normal rabbit serum was 2% (median), with a range of zero to 11%. These values have not been subtracted from those obtained after incubation with the antithymocyte serum.

**RESULTS**

The largest single group of specimens with T-cell properties were the eight specimens in which blasts bound sheep erythrocytes under all conditions employed and expressed T-cell antigens (Table 1). The proportions of cells capable of forming rosettes with untreated sheep erythrocytes at 37°C ranged from 4% to 71% (median 44%). Incubation at 4°C resulted in the same proportions of E-positive cells (median 45%) as obtained at the higher temperature. This group, therefore, corresponds to the E-positive ALLs that are widely recognized by rosette formation with untreated sheep erythrocytes. With AET-treated erythrocytes, even larger proportions of blasts in these specimens were capable of forming rosettes. The proportions of rosette-forming cells increased to a median value of 72%, with a

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<th>Specimen Number</th>
<th>Percentage Rosette-Forming Cells at 37°C</th>
<th>Percentage Rosette-Forming Cells at 4°C</th>
<th>Percentage Cells With T-Antigens With AET-Treated E*</th>
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<tr>
<td>1</td>
<td>4</td>
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\(^*\)Sheep erythrocytes.
range of 14%–93%. In all eight specimens, T-cell antigens were present on some proportion of the cells, although the percentages of positive cells by immunofluorescence were not consistently related to the values obtained with the rosette assays.

Among the blast specimens that failed to form rosettes with untreated sheep erythrocytes at either 37°C or 4°C, two additional groups with T-cell properties could be identified. The numbers of specimens in these groups exceeded those that constitute the usual E-positive group. A group of four specimens expressed T-cell antigens on more than one-half of the cells, even though E-rosette formation could not be demonstrated by any of the assays used (Table 2). A few additional specimens had lower proportions of cells reactive with the antithymocyte serum, but it was not possible to determine whether these reactive cells were morphologically normal T cells or blast cells. Other studies of ALL populations have also identified blast samples that were E-negative but expressed T-cell antigens.35,416

The third group was identified as expressing a combination of membrane markers intermediate between the two previously identified patterns (Table 3). These six additional specimens were identified by their ability to bind AET-treated erythrocytes. The proportions of blasts forming rosettes with treated erythrocytes had a median of 41% (range 3%–58%) and were generally lower than those observed for the E-positive group (Table 1). The expression of T-cell antigens on cells in these specimens was variable. Two samples (specimens 15 and 17) had T-cell antigens on nearly all cells; two (specimens 13 and 16) had T-cell antigens on approximately the same proportions of cells as formed rosettes; two (specimens 14 and 18) lacked detectable T-cell antigens.

A total of 18 (25%) of the 72 leukemia specimens could be identified as having membrane feature(s) indicative of T-cell differentiation by this combination of rosette assays and serologic testing.

### Table 2. Frequency of Cells With T-Cell Properties: Lymphoblast Specimens That Expressed T-Cell Antigens Only

<table>
<thead>
<tr>
<th>Specimen Number</th>
<th>Percentage Rosette-Forming Cells</th>
<th>Percentage Cells With T Antigens</th>
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<tbody>
<tr>
<td></td>
<td>at 37°C</td>
<td>at 4°C</td>
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<tr>
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<td>1</td>
<td>2</td>
</tr>
<tr>
<td>10</td>
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<tr>
<td>12</td>
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*Sheep erythrocytes.

### Table 3. Frequency of Cells With T-Cell Properties: Lymphoblast Specimens That Formed Rosettes Only With AET-Treated Sheep Erythrocytes (E)

<table>
<thead>
<tr>
<th>Specimen Number</th>
<th>Percentage Rosette-Forming Cells</th>
<th>Percentage Cells With T Antigens</th>
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DISCUSSION

In view of the variation in estimates of the incidence of T-cell ALL and because of the different methods used in their identification, the present study applied several of the commonly used tests to each bone marrow specimen from 72 pediatric patients diagnosed as having ALL.

The data show that of the methods used, only two, namely rosetting with untreated sheep erythrocytes at 37°C and at 4°C, gave consistently concordant results. When AET-treated sheep erythrocytes were used, both the numbers of reacting specimens and the proportions of rosette-forming blast cells increased. Since the increases in the numbers of cells rosetting with AET-treated erythrocytes over the numbers rosetting with untreated erythrocytes varied between specimens from different patients, it seems reasonable to assume that AET treatment does not just enhance the sensitivity of the test but probably activates sites on the red cells that are recognized by additional numbers of blast cells. It is noteworthy that the specificity of AET-treated-erythrocyte rosetting by T cells was recently questioned when this property was found in a B-lymphoid cell line. In our series, two ALL specimens (specimens 9 and 10) that formed rosettes with treated sheep erythrocytes, but not with untreated sheep erythrocytes reacted with anti-ALL serum and an anti-Ia-like serum and lacked detectable surface Ig, thus resembling common-type ALL cells. These findings suggest that any increase in sensitivity achieved through the use of AET-treated erythrocytes is offset by a decrease in specificity.

There exists ample evidence that antigenic markers reactive with anti-T sera are not identical with E receptors. Our anti-T serum was shown not to inhibit E rosetting by normal blood lymphocytes. Thus discrepancies between the results of E rosetting and tests for T-cell antigens are not surprising.

Taken as a whole, the results of this study suggest that in addition to the patients whose blast cells present serologic markers and E-rosetting properties similar to those of normal thymocytes, there are others whose blast cells may resemble normal T cells in various stages of differentiation. E-negative blasts that express T-cell antigens have been hypothesized to be a malignant counterpart of a normal prethymus cell. The existence of a group of blasts with intermediate properties suggests a sequence in which early cells in the T lineage acquire T-cell antigens, then the ability to form rosettes only under the most favorable conditions, as indicated by rosette formation with AET-treated erythrocytes, followed by acquisition of the ability to form rosettes under all conditions. Mature peripheral T cells are characterized by temperature-sensitive rosetting and loss of a thymus antigen.

E-positive blast specimens identical to those expressing multiple T-cell properties in this study are associated with poor prognostic clinical features at diagnosis and poor clinical course. Since not all blasts possess the full complement of surface markers generally associated with T-cell leukemia, the degree of correlation of cell surface markers with clinical features and with clinical course may be directly related to the method used to type for T-cell properties. It remains to be determined if patients whose blasts express fewer T-cell-associated features will have a similarly poor clinical course or a course intermediate between that of the usual T-cell leukemia and that of the common ALL. The prognostic values of these several markers will be established by long-term follow-up of such patients.
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