Lymphoblasts With Both T and B Markers in Childhood Leukemia and Lymphoma

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A modification of the double rosette method of Mendes et al. was used to examine lymphoblasts from 22 children with acute lymphoblastic leukemia and 1 child with lymphoblastic lymphoma. This method was used since it directly and simultaneously detected T, B, and null lymphocytes as well as monocytes. In addition it detected the D lymphocyte which has both T- and B-cell surface markers. Based on surface characteristics, lymphoblasts from the patients studied could be divided into three groups: (1) null cells with no detectable markers; (2) cells with T-cell markers; or (3) D or double cells with both the T-cell receptor for sheep erythrocytes and the B-cell receptor for activated complement. No lymphoblasts were found which had easily detectable surface immunoglobulins or complement receptors alone. Eighteen patients had null-cell lymphoblasts; four patients, including the child with lymphoma, had two lymphoblast populations, T and D cells. No circulating D lymphoblasts were detected when these patients went into remission. This simple, reproducible method utilizes easily prepared, stable reagents for surface-marker detection and has the advantage of detecting directly another lymphoid subpopulation, the D cell.

CELL SURFACE MARKERS have been used to classify lymphoproliferative malignancies as T or B cell in origin. T lymphocytes (50%-75% of normal human blood lymphocytes) are identified by their specific surface antigens and by their ability to form rosettes with sheep erythrocytes (E). B lymphocytes (5%-20% of blood lymphocytes) are identified by several markers: surface immunoglobulins, receptors for complement (C), and receptors for the Fc portion of IgG. Lymphocytes (5%-10%) with no detectable surface markers are also present in normal blood; these are called null cells. Recently, lymphocytes with both T- and B-cell markers, the double or D cell, have also been found in normal blood (less than 5%).

On the basis of surface markers, lymphoblasts from children with acute lymphoblastic leukemia (ALL) are either null cells or T cells. Null-cell ALL appears to have a less aggressive clinical course and a better prognosis than T-cell ALL.

We report here the further heterogeneity of childhood ALL in which morphological lymphoblasts from some patients had both both T- and B-cell surface markers. Recently, several cases of childhood lymphoblastic lymphoma were reported in which the lymphoblasts also bore both T- and B-cell mark-
In our investigation, surface markers were assessed simultaneously by our modification of the double rosette method of Mendes et al. This modified method is a clinically useful tool for examining the surface markers of normal and malignant lymphoid cells.

**MATERIALS AND METHODS**

**Patients**

The patients were 22 children with lymphoproliferative malignancies: 21 had ALL; 1 had lymphoblastic lymphoma involving the mediastinum. The diagnosis of ALL was made on the basis of morphological and cytochemical examinations of the bone marrow and peripheral blood. The patients with ALL represented 21 consecutive new admissions at several Bay Area hospitals. They were studied at the time of diagnosis and were entered into the treatment protocol of the Children’s Cancer Study Group. Blood and marrow cells of patients EO and VT were studied at time of diagnosis, when remission was induced, and during remission. Blood and tumor cells from the mediastinal mass of patient LM were studied at the time of diagnosis; blood lymphocytes were examined during remission. Normal blood lymphocytes were studied from 40 healthy adult volunteers and 5 normal children. Appropriate informed consent was obtained for these studies.

**Purification of Lymphoid Cells**

Lymphocytes or lymphoblasts from heparinized (100 U/ml) venous blood were isolated by the method of Böyum. They were washed and resuspended to a final concentration of $5 \times 10^6$ cells/ml in RPMI-1640 tissue culture medium containing $10\%$ fetal calf serum (FCS). Cells from heparinized bone marrow aspirates were dispersed from spicules by repeated aspiration through a Pasteur pipette. Contaminating red cells were lysed with $0.83\%$ NH$_4$Cl, and the bone marrow leukocytes were washed and resuspended to a final concentration of $5 \times 10^6$ cells/ml in medium.

Tissue from the mediastinal mass from patient LM was finely minced and a cell suspension was made and purified using the same procedure as for the bone marrow cells. Viability as assessed by trypan blue dye exclusion was greater than $95\%$ for the blood and marrow cells and $70\%$ for the biopsy cells.

**Cell Surface Receptor Studies**

The purified mononuclear cells ($5 \times 10^6$ cells/ml) were examined by both separate and simultaneous rosette assay systems.

**T-cell detection.** T cells were identified by rosette formation with untreated E$^3$ or with neuraminidase-treated E.$^23$ A rosette was defined as any lymphocyte or "blast" having three or more erythrocytes attached to its surface. In all rosette studies, 200 viable cells were counted in duplicate in the presence of trypan blue.

**B-cell detection.** B cells were identified by the presence of the C receptor$^5,24$ or surface immunoglobulin.$^4,25$ The complement receptor was detected by rosette formation with either EAC (erythrocytes sensitized with rabbit anti-E (IgM) and mouse complement)$^5$ or zymosan complement (ZC) particles.$^{11,26}$ ZC was prepared by incubating equal volumes of zymosan particles ($2 \times 10^9$/ml) and fresh mouse complement (1:20 dilution in gelatin Veronal buffer) at $37^\circ$C for $1/2$ hr. These prepared particles could be stored in small aliquots at $-70^\circ$C and thawed immediately before use. Mononuclear cells (0.1 ml) were incubated by rotation at $37^\circ$C for $1/2$ hr with 0.1 ml EAC or ZC ($1 \times 10^7$/ml). An EAC rosette had three or more attached red cells; a ZC rosette had two or more particles attached.$^{26}$ With blood and marrow lymphoid cells both reagents gave comparable results and only ZC was used in the simultaneous system. Surface immunoglobulin bearing cells were determined by direct immunofluorescence with fluorescent conjugates of anti-human IgA, G, or M.$^4,25$

**Monocyte detection.** Monocytes were identified by Fe$^{27,28}$ and complement$^{29}$ receptors, phagocytosis and cytochemical determination for nonspecific esterase activity.$^{30,31}$ The monocyte Fe receptor was detected by rosette formation and phagocytosis of 7S EA (sheep red cells sensitized with anti-sheep red cell IgG). Monocytes and red cells were incubated by rotation at $37^\circ$C for $1/2$ hr.
LYMPHOBLAST IDENTIFICATION

Rosette formation and phagocytosis were assessed in a hemocytometer as well as on cytocentrifuge preparations. The C receptor was detected as above. Phagocytic properties of monocytes were also studied with zymosan, heat-killed Candida, latex (0.81 μm in diameter), and heat-killed Staphylococcus aureus 502A. Particle-to-cell ratio was 20:1 to 100:1 and cells were rotated with particles at 37°C for 1 hr.

Simultaneous detection system. The simultaneous detection of T and B cells and monocytes was determined by a modification of the method of Mendes and colleagues. Neuraminidase-treated E were used instead of untreated E. Neuraminidase treatment resulted in more stable rosettes and the treated E could be stored up to 4 wk at 4°C. ZC particles were prepared as above. The actual assay consisted of the following steps: 0.1 ml isolated mononuclear cells (5 × 10⁶ cells/ml) was incubated with 0.1 ml neuraminidase-treated E (10⁶ cells/ml) and 0.05 ml ZC (10⁸ particles/ml) at 37°C for 15 min. The suspension was centrifuged at 1000 rpm for 10 min and refrigerated overnight. Cells were gently resuspended by inversion. Enumeration of cell subpopulations was done in the presence of trypan blue, which allowed discrimination between viable and nonviable cells and which stained the zymosan particles blue. Rosettes were defined by the same criteria as in the single-assay system. A double-rosetted D cell had at least three E and two ZC attached to its surface. A null cell had no attached or ingested particles. Monocytes were defined as cells which had phagocytosed at least one ZC particle. Wright Giemsa stained cytocentrifuge preparations were made to assess the morphology of the rosetted cells.

RESULTS

Normal Control Studies

The percentages of blood B and T cells of normal adult controls are tabulated in Table I. These results confirmed those of Mendes et al. who found the same percentage of B and T cells whether determined separately or simultaneously. The same number of C receptors was detected whether they were examined with the EAC or ZC reagent. Since zymosan directly activates complement, specially prepared antibodies to zymosan were not needed. The elimination of antibody from this procedure assured that the C receptor and not the Fc receptor for IgG was detected. The average percentage of D cells in normal peripheral blood was 0.7± with a range of 0% to 2%, and no greater than 2% was found in our normal controls.

Figure 1 is a composite photomicrograph which shows T, B, and D cells as visualized in a hemocytometer chamber.

An average of 4% monocytes was found by the double-rosette method as determined by phagocytosis of the ZC reagent. Only 2% of the mononuclear cells formed rosettes with the 7S EA under the conditions used. In separate assays, using phagocytosis of latex beads, a mean of 11% monocytes was found. Examination of cytocentrifuge slide preparations, stained either with Wright

| Table 1. Mononuclear Cell Subpopulations in Normal Peripheral Blood (Percent) |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| Separate Assays             | Double-Rosette Method       |                             |                             |                             |
| T                           | 67.6                        | 65.6                        | 61.4                        | 65.6                        |
| B*                          | 14.8                        | 11.4                        | 4.3                         | 21.9                        |
| B+                          | 2.1                         | 1.3                         | 0.2                         | 0.67                        |
| B-                          | 2.1                         | 1.3                         | 0.5                         | 2.1                         |
| M1                          | 40                          |                             |                             |                             |
| M2                          | 2.1                         |                             |                             |                             |
| N1                          | 2.1                         |                             |                             |                             |
| N2                          | 2.1                         |                             |                             |                             |

*B-cell complement receptor determined with EAC.
†B-cell complement receptor determined with ZC.
1Monocytes as phagocytic cells.
§Null cells.
Fig. 1. (A) Normal viable lymphocytes which have formed rosettes with neuraminidase-treated sheep erythrocytes, T cell (top) and complement-coated zymosan particles, B cell (arrow). Trypan blue 0.1%. (B) Normal D lymphocyte with both sheep erythrocytes and zymosan particles (arrow).

Giemsa or for nonspecific esterase activity, \(^{30,31}\) resulted in a higher percentage, 14\(^\circ\) to 18\(^\circ\), of detectable monocytes than either of the rosetting procedures. Null cells in the double-rosette system ranged from 5\(^\circ\) to 15\(^\circ\). Surface immunoglobulin-bearing cells were not detected in this system and enumeration of those cells would probably have decreased the number of calculated null cells.

Patient Studies

Initial clinical features of the children studied were as follows (Table 2): The patients were divided into two groups: those with null-cell lymphoblasts (I), and those with both T- and D-cell lymphoblasts (II). Four children from group I presented with mediastinal masses and one of these 4 patients had a white blood cell count greater than 100,000, as did 3 other children without mediastinal masses. Seventeen of 21 patients with bone marrow evidence of leukemia had 50\(^\circ\), or more blasts in the blood. All 4 children in group II had mediastinal masses, 3 had white cell counts above 100,000, and 1 child, LM, had no evidence of leukemia in the bone marrow or blood. All 4 had easily inducible first remissions. The percentages of null and D cells detected in the blood of all patients at time of diagnosis are also presented in Table 1. Of the 18 patients with null-cell ALL all but patient MJ had greater than 40\(^\circ\), null cells in the blood. Patient MJ had only 8\(^\circ\), "blasts" in his blood and more than 40\(^\circ\), morphologically normal blood lymphocytes. No patient had blasts with only C receptors.

Patients in group II were studied further when remission was induced and during remission. These data are presented in Table 3. Bone marrow and blood cells were examined by both the separate and the simultaneous assay systems. Cells from biopsy tissue from patient LM were also studied. In all cases the
Table 2. Initial Clinical Features of Children Studied

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<th>Patient</th>
<th>Age (yr)</th>
<th>Sex</th>
<th>Mediastinal Mass</th>
<th>WBC 10^9/liter</th>
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<th>Percent Blood D Cells</th>
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<td>9.6</td>
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* Those with null-cell lymphoblasts.
† Those with both T- and D-cell lymphoblasts.
‡ Patient with mediastinal lymphoma.

Table 3. Surface Markers on Lymphoid Cells (Percent) From Patients With T- and D-Cell Lymphoblasts

<table>
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<tr>
<th>Patient</th>
<th>Stage of Disease</th>
<th>Cells</th>
<th>Separate Assays</th>
<th>Double-Rosette Assay</th>
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<td>EO</td>
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<td>Blood</td>
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<td>$E_R$ $C_R$ $D(E_R &amp; C_R)$</td>
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<tr>
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<td>Diagnosis</td>
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<td>$E_R$</td>
<td>$E_R$ $C_R$ $D(E_R &amp; C_R)$</td>
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results from both methods were similar in total number of E-rosette-forming cells \((E_R)\) and C-receptor cells \((C_R)\). At the time of diagnosis, most blood and marrow “blasts” from patients EO, VT, and MS formed \(E_R\)—suggestive of T-cell ALL. However, \(10^{5}\), \(20^{5}\) of these \(E_R\) blasts also had complement receptors; hence, they were D cells. When these “blasts” were further examined by direct immunofluorescence, no surface immunoglobulin was detected.

Most lymphoblasts isolated from the mediastinal mass from LM formed \(E_R\); \(15^{5}\) were also D cells with no detectable surface immunoglobulin. Because lymphoblastic lymphoma frequently progresses to leukemia,\(^2\) blood from patient LM has been examined at frequent intervals to detect the advent of leukemic infiltration. To date, 23 mo after diagnosis, no morphological lymphoblasts or increased numbers of D cells have appeared in the blood. At remission, normal values for D cells were found in blood and bone marrow of all four patients. All patients are still in their first remission, which was induced in 4 wk or less. Lengths of remission to date are LM, 24 mo; EO, 19 mo; VT, 10 mo; and MS, 5 mo.

**DISCUSSION**

The presence of cell surface markers and differentiation antigens has facilitated the characterization and classification of mononuclear cells in many lymphoproliferative malignancies. The advantage of the double-rosette method of Mendes et al.\(^1\) for detection of cell surface markers is that monocytes and four lymphocyte subpopulations can be identified simultaneously. Rosette formation with sheep erythrocytes is specific for T cells. Unfortunately, other surface markers are not as cell specific and B lymphocytes and monocytes have several surface markers in common. These two cells can be differentiated with the method used in the present study by monocyte ingestion of the ZC particles: \(4^{5}\), \(10^{5}\) monocytes have been found by this method, similar to the values found by Robinson and Lertrutanakul\(^7\) using latex ingestion for monocyte identification.

Latex ingestion and several other techniques to identify monocytes also were used in our laboratory. A wide variation, \(4^{5}\), \(25^{5}\) of detectable monocytes, was found depending on the test system used. Observations by phase microscopy of ingestion of IgG-coated red cells or latex confirmed the percentages found by the double-rosette technique. On the other hand, when cytocentrifuge slide preparations were made, \(15^{5}\), \(25^{5}\) monocytes were detected. This frequency was found whether the monocytes were identified by phagocytic properties or by nonspecific esterase activity. Cytocentrifugation appeared to produce artifacts in terms of the number and distribution of the various cell types and should only be used for morphological examination of the cells and not enumeration of cell subpopulations.

Thus, the adaption of the method of Mendes et al. allows the simultaneous discrimination of monocytes and four lymphoid subpopulations, and data obtained by the technique are comparable to those obtained by the more commonly used single methods.

The use of this method to study lymphoblasts from children with ALL and lymphoblastic lymphoma had the advantage of detecting complement receptors
as well as T and null cells. Previous large studies of surface markers in childhood ALL have reported the leukemic cells to be either null or T cells.\textsuperscript{14, 19} The presence of the complement receptors was not investigated in most studies,\textsuperscript{2, 12, 14, 16, 18, 19} and when examined it was not detected simultaneously with E-rosette formation.\textsuperscript{33} In our series no lymphoblasts with only complement receptors or surface immunoglobulin were detected. However, a significant percentage of T-cell lymphoblasts from four patients had complement receptors. T-cell lymphoblasts with complement receptors (D lymphoblasts) previously have been reported in several patients with lymphoblastic lymphoma.\textsuperscript{20, 21} All of those patients had mediastinal masses and one adult patient had a leukemic conversion and died 24 mo after diagnosis. It had been suggested previously that lymphoblastic lymphoma of the T-cell type shared many clinical and immunologic features with T-cell ALL\textsuperscript{34, 36} and may represent different clinical manifestations of the same malignant process. The recent reports of lymphoblastic lymphoma with D cells and the finding of D cells in ALL suggested another similarity between lymphoblastic leukemia and lymphoma.

The origin and function of normal D cells is unknown. It has been speculated that they may be uncommitted stem cells with the capacity of developing into either B or T cells.\textsuperscript{37} The D cell could be a dedifferentiated cell or a lymphocyte early in its differentiation. Stein et al.\textsuperscript{21} have suggested that the D cell found in lymphoblastic lymphoma is similar to a fetal thymocyte which is acid-phosphate positive with complement receptors. However, in the in vitro study of human fetal thymocytes there has been no demonstration of both E-rosetting and complement receptors on one cell.\textsuperscript{38} The D cell may also represent a sub-population of T lymphocytes since there is increasing evidence that subpopulations of T cells can possess B-cell surface receptors.\textsuperscript{39, 42} Thus, both the origin and function of the normal D cell require further investigation. D cells have also been reported in several isolated cases of leukemia.\textsuperscript{43, 46}

The clinical significance of D cells in our four patients, three with ALL and one with lymphoblastic lymphoma, is also not known. The presence of these cells at the time of diagnosis and their absence during remission may give some clue as to the pathogenesis of the disease. Elevated D cells may, in fact, be specific for lymphoblastic malignancies of the T-cell type since they have not been found among the malignant T cells of Sezary syndrome (three cases) or the B cells from ten patients with chronic lymphocytic leukemia (unpublished observations). If D cells increase upon relapse they may be useful prognostic indicators and may help assess the efficacy of the treatment. All four of the patients reported here are still in their first remission.

The clinical value of D cells may be assessed only after a large number of cases has been studied over a longer period of time. The simultaneous detection method reported here can be applied easily to all new cases of ALL and other lymphoproliferative malignancies and may help characterize further these diseases and thereby lead to more objective diagnoses and effective treatment.

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