Immunologic Classification of Acute Lymphoblastic Leukemia: Implications for Normal Lymphoid Differentiation

By Kenneth A. Foon, Ronald J. Billing, Paul I. Terasaki, and Martin J. Cline

Acute lymphoblastic leukemia (ALL) is a heterogeneous disease as defined by clinical characteristics and immunologic techniques. The standard cell surface markers are sheep erythrocyte receptors for T lymphocytes and surface membrane immunoglobulin for B lymphocytes. Utilizing these markers, three subtypes of ALL designated T-ALL, B-ALL and non-B, non-T or null ALL have been defined. We have studied 70 patients with ALL utilizing these standard cell surface markers. In addition, we have further subclassified these patients by testing each cell for an ALL-associated antigen, the la-like antigen, and thymocyte antigen(s) all defined by well-characterized antisera. We can define 12 subgroups of ALL by their surface antigenic characteristics. These subgroups may have relevance to the clinical expression of disease and may define identifiable stages of normal lymphocyte development.

ACUTE lymphoblastic leukemia (ALL) is a heterogeneous disease. This heterogeneity has been defined by clinical characteristics and by immunologic techniques. The standard cell surface marker studies for normal B and T lymphocytes that have generally been used to define the subtypes of ALL include sheep erythrocytes (E) receptors on T lymphocytes and surface membrane immunoglobulin (SmIg), complement (C'), and Fc receptors on B lymphocytes. The three major subtypes of ALL as defined by these tests are designated T-ALL, B-ALL and non-B, non-T-ALL.

Patients with ALL cells devoid of any of the classical T and B markers (i.e., non-B, non-T) comprise 70%-80% of the total cases of ALL. Patients with E-rosette-positive cells (i.e., T-ALL) constitute 20%-39% of cases. B-derived ALL, defined by cells with SmIg and other standard B-lymphocyte surface membrane markers, is the least common, representing only 1%-3% of patients with ALL.

Recently, heteroantisera commonly raised in rabbits to normal and malignant lymphocytes have demonstrated considerable heterogeneity within the B- and T-lymphocyte populations defined by standard surface markers. Three such heteroantisera are of particular interest for the classification of ALL and understanding its pathogenesis: (1) antisera reacting with the common region of the HLA-DR (la-like) antigen, (2) antisera reacting with antigens specific to T lymphocytes and that may define subgroups of T-ALL that are E-rosette negative, and (3) antisera reacting with the "ALL-associated" or "common-ALL" antigen found on approximately 70% of non-B, non-T-ALL, and rarely on B- and T-ALL.

In this report we describe 12 subgroups of ALL defined by standard surface markers and these 3 serologically detected surface antigens. If one assumes that, in a given patient, ALL results from proliferation of a malignant clone derived from a single cell arrested at a specific stage of lymphoid differentiation, then analysis of the various subgroups of ALL should provide a means of elucidating the surface markers that characterize normal lymphoid differentiation.

TECHNOLOGY OF SURFACE MARKER ANALYSIS

The diagnosis of ALL was based on clinical features, Romanowsky-stained peripheral blood and bone marrow samples, and special cytochemical studies including myeloperoxidase, periodic acid-Schiff, and neutral lipid stains. Peripheral blood or bone marrow samples were obtained in heparinized syringes and prepared on a Ficoll-Hypaque gradient. Test cells were >80% leukemic blasts.

SmIg was detected with a polyvalent heavy-chain antisemur prepared in goats (Meloy Laboratories, Inc., Springfield, Va.). Positive cells were further tested with anti-IgG, anti-IgM, anti-kappa, and anti-lambda sera. Fifty microliters of leukemia cells at 1 × 10^6 per ml were mixed with 5 μl of fluorescein-labeled antisemur and allowed to incubate 30 min at 4°C. The cells were then washed, and the number of labeled cells determined by ultraviolet microscopy. The designation of B-ALL was reserved for patients with greater than 25% SmIg-labeled blasts.

E-rosette-forming cells were detected by adding 20 μl of neuraminidase-treated SRBC (1 × 10^8 per ml) to
IMMUNOLOGIC CLASSIFICATION OF ALL

20 μl of leukemia cells (5 × 10^6 per ml) and centrifuging for 5 min at 500 g. The cells were incubated for 30 min at 5°C, gently resuspended, and 200 cells were counted for rosettes.

The rabbit heteroantisera used in these studies have been previously described. An antiserum with Ia-like specificity was produced by immunization of rabbits with papain digests of cell membranes of involved spleens from patients with a variety of hematologic malignancies. An antiserum with T-cell specificity was prepared by intravenous immunization of a New Zealand white rabbit with human thymus cells and absorbed with an Epstein-Barr virus (EBV)-transformed lymphoblastoid cell line. An antiserum that identified an ALL-associated cell membrane antigen was raised in rabbits to non-B, non-T-ALL cells and identified an ALL-associated cell membrane antigen. An antiserum that formed lymphoblastoid cell line. An antiserum that was raised in rabbits to non-B, non-T-ALL cells and identified an ALL-associated cell membrane antigen.

A microcytotoxicity test was performed by the addition of 1 μl of antiserum at various dilutions to 1 μl of cell suspension (2000 cells) in a Microtest plate. The mixture was incubated at 22°C for 30 min, then 5 μl of rabbit complement was added for an additional 60 min. The reaction was then stained with eosin dye, fixed, and read for cytotoxicity. Positive reactions were >80% nonviability of cells. Titers of positive reactions ranged from 1:16 to 1:256. Negative reactions were less than 30% nonviability of cells in undiluted serum.

SUBCLASSIFICATION OF ALL

ALL cells from 70 patients representing children and adults were tested for 5 different surface membrane markers. From these data we were able to differentiate 12 subgroups within the 3 major groups of ALL patients (Table 1).

Three non-B, non-T subgroups were described and comprised 80% of the total number of patients. Subgroup I had no detectable surface markers including Ia-like and the ALL-associated antigens. Subgroup II cells expressed only the Ia-like antigen. Subgroup III was the most common subgroup and these cells were devoid of classical T and B markers but had Ia-like and ALL antigens. Approximately 20% of these cells would probably be classified as pre-B ALL (subgroup X) if they were tested for intracytoplasmic IgM.

Six subgroups of T-derived ALL were identified. They were considered T lymphocytes as they had E receptors and/or a thymocytes antigen(s). Subgroups IV through VII had the thymocyte-related antigen but did not have receptors for sheep erythrocytes. This finding has been previously reported and emphasizes the importance of utilizing well-characterized heteroantisera that define T antigens, in addition to testing for E rosettes. Otherwise, many T-derived cells would be classified as "null" cells on the basis of non-E rosetting. In these cases with cells that were T+ and E-, the Ia-like and ALL antigens were present in various combinations and were the primary determinants in classifying the four subgroups.

Subgroups VIII and IX had typical T lymphoblasts that formed E rosettes and had T antigens. These subgroups differ from each other in the sense of expressing the thymocyte antigen(s). Subgroups VIII also had Ia-like antigens. Neither of these subgroups had the ALL antigen. In no case did we observe cells that were E+ and T-.

Subgroup X is a pre-B-cell ALL, defined by intracytoplasmic IgM. These cells were reported to have Ia-like antigens and the "common" ALL antigen. We did not test for intracytoplasmic IgM; therefore, no patients are included in this subgroup. In previous studies, in which these pre-B cells were described,

Table 1. Immunologic Classification of ALL

<table>
<thead>
<tr>
<th>Group</th>
<th>Subgroup</th>
<th>Markers</th>
<th>No. (%)</th>
<th>Sex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-B</td>
<td>I</td>
<td>Smg'Ia'ALL''E''T''</td>
<td>2/70 (3)</td>
<td>M</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>Smg'Ia'ALL''E''T''</td>
<td>9/70 (13)</td>
<td>F</td>
</tr>
<tr>
<td></td>
<td>III*</td>
<td>Smg'Ia'ALL''E''T''</td>
<td>45/70 (64)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IV</td>
<td>Smg'Ia'ALL''E''T''</td>
<td>1/70 (1.5)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>V</td>
<td>Smg'Ia'ALL''E''T''</td>
<td>1/70 (1.5)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>VI</td>
<td>Smg'Ia'ALL''E''T''</td>
<td>1/70 (1.5)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>VII</td>
<td>Smg'Ia'ALL''E''T''</td>
<td>3/70 (4)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>VIII</td>
<td>Smg'Ia'ALL''E''T''</td>
<td>2/70 (3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IX</td>
<td>Smg'Ia'ALL''E''T''</td>
<td>4/70 (5.5)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>X</td>
<td>Smg'CigM*Ila'ALL''E''T''</td>
<td>— —</td>
<td></td>
</tr>
<tr>
<td></td>
<td>XI</td>
<td>Smg'Ia'ALL''E''T''</td>
<td>1/70 (1.5)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>XII</td>
<td>Smg'Ia'ALL''E''T''</td>
<td>1/70 (1.5)</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td></td>
<td></td>
<td>2/70 (3)</td>
<td></td>
</tr>
</tbody>
</table>

*Cytoplasmic IgM was not tested for in this study and therefore 20% of this group may in fact be pre-B ALL (Subgroup X).17-29
approximately 20% of the non-B, non-T-ALL were found to actually be pre-B cells. Subgroups XI and XII are B derived, defined by SmIg demonstrating monoclonal IgM lambda and IgM kappa, respectively. Both of these cells had Ia-like antigens while subgroup XI also had the ALL antigen.

NORMAL LYMPHOID MATURATION

Figure 1 outlines a hypothetical scheme of normal human lymphoid cell maturation from the pluripotent stem cell through differentiation to mature B and T lymphocytes. Surface membrane markers are indicated at each level of maturation. Many of these markers are well defined, while others are inferred from the markers of corresponding ALL cells.

There is good evidence that the Ia-like antigen is a normal differentiation antigen. While it is not found on mature granulocytes, it is detected on myeloid progenitor cells by both cytotoxicity and the fluorescent activated cell sorter (FACS). Furthermore, Greaves and Janossy have reported lymphoid cells that are Ia⁺ but SmIg⁻ and cells that resemble myeloblasts and are Ia⁺ in fetal marrow and the normal marrow of children by FACS analysis.

There is also substantial evidence that the ALL antigen is also a normal differentiation antigen. In addition to ALL cells, it has been detected on acute undifferentiated leukemia cells, lymphoma cells, and chronic myelogenous leukemia-blast crisis cells. More important, it is not found on any normal mature hematopoietic cells, but it has been identified on normal fetal liver cells and normal bone marrow cells from children. These cells were isolated on a FACS and were non-B, non-T "lymphoid" cells and commonly also had the Ia antigen. Thus, the presence of Ia-like and ALL antigens on ALL cells probably reflects arrest at specific levels of differentiation. Greaves and Janossy, who pioneered this field, have expressed a similar viewpoint.

PLURIPOTENT STEM CELL

There is no widely accepted assay for the pluripotent hematopoietic stem cell in man. A recently described in vitro assay for multipotential human stem cells has been described, but there is as yet insufficient evidence to conclude that it is the precursor of both myeloid and lymphoid cell lines. Consequently, the antigenic characteristics of the pluripotent stem cell can be inferred only from experience with human bone marrow transplantation. This type of indirect evidence suggests that the ALL antigen is not present on the pluripotent stem cell. For example, remission
bone marrow from a patient with ALL-antigen-positive leukemia was treated with antiserum to the “common” ALL antigen in vitro prior to cryopreservation.\textsuperscript{28} When the patient’s disease relapsed, he was treated with intensive chemotherapy and 1000 rads of total body irradiation and transplanted with his own cryopreserved, antiserum-treated bone marrow. Reconstitution of all hematopoietic cell lines, including lymphocytes, ensued. This suggested that the pluripotent stem cell did not express the ALL antigen.

There are no data in man as to the presence of the Ia-like antigen on the pluripotent stem cell. The Ia-like antigen has been reported to be expressed by early myeloid\textsuperscript{30-33} and erythroid\textsuperscript{34} progenitor cells, as well as by monocytes,\textsuperscript{5} B lymphocytes,\textsuperscript{2} a small percentage of normal T lymphocytes\textsuperscript{50} and immature T lymphocytes,\textsuperscript{31} and most activated T lymphocytes.\textsuperscript{32} Since this antigen is shared by myeloid, erythroid, and lymphoid cells, one might predict that it would be present on the pluripotent stem cell common to all three lines. However, data from the mouse suggest that murine pluripotent stem cells lack Ia-antigens.\textsuperscript{33} We must conclude that for the present the Ia-antigen status of the pluripotent stem cell is uncertain. We would not expect the markers acquired with differentiation and found on defined B and T lymphocytes such as T-antigens, E-rosettes, and SmIg to be present on the pluripotent stem cell.

The data presented in Table 1 suggest that subgroup I ALL is closest in lineage to the pluripotent stem cell. This conclusion is based on the fact that the subgroup I ALL cells lack all of the surface membrane markers, including Ia-like and ALL antigens.

**COMMITTED LYMPHOID PRECURSOR**

Like the pluripotent stem cell, there is no known assay for the committed lymphoid precursor cell. We would not expect this cell to have T antigens, E receptors, or SmIg, which distinguish more mature T and B lymphocytes. However, we might expect this cell to have differentiation antigens such as the Ia-like and ALL antigens which are found on less mature B and T lymphocytes. The Ia-like antigen is common to both T and B cells and would therefore be expected to be present on their common precursor cell. In addition, the ALL antigen is occasionally found on more differentiated T and B lymphoblasts (subgroups IV, V, and X) and also might be present on the committed precursor of both lines of lymphoid differentiation. An ALL cell with these surface marker characteristics exists in subgroup III. This is the most common type of non-B, non-T-ALL and is Ia\textsuperscript{+} and ALL\textsuperscript{+}, but lacks all other classical surface markers. We propose that in ALL this malignant cell may be derived from the committed lymphoid precursor. In normal human bone marrow from fetuses and children, a “lymphoid” cell has been identified that has the identical phenotype (Ia\textsuperscript{+}, ALL\textsuperscript{+}, SmIg\textsuperscript{-}) as the subgroup III ALL cell.\textsuperscript{24}

Subgroup II is also a non-B, non-T cell in that it lacks classical B and T markers. The only surface marker detected is the Ia-like antigen. Subgroup II may be a transitional cell between the pluripotent stem cell and the committed lymphoid precursor. Alternatively, it may reflect lack of differentiation antigen as a consequence of aberrant maturation.

**PRE-T LYMPHOCYTE**

There is considerable evidence that lymphocytes destined to become functional T cells express T antigens at an early stage of fetal development\textsuperscript{34-36} and may express some T-cell antigens prior to migration to the thymus.\textsuperscript{34-36} Ia-like antigens have also been described on immature T cells.\textsuperscript{31} Most of the functional properties of T cells,\textsuperscript{34-36} and E receptors probably develop later, after thymic priming.

The pre-T cell might then be a cell with T antigens but lacking E receptors. It may or may not express normal differentiation antigens such as the Ia-like and ALL antigens. Subgroups IV through VIII would all be linked to these, as they have the T antigen(s) and various combinations of Ia-like and ALL antigens.

**EARLY T LYMPHOCYTE**

As the T lymphocyte matures it no longer expresses the ALL antigen. Although the Ia-like antigen is probably present in low quantity, it is rarely detected by routine testing. At this point the cell has probably gained the E receptor which has been reported to be stable at both 4\textdegree{}C and 37\textdegree{}C.\textsuperscript{41} The ALL subgroups VIII and IX are closest in origin to this cell.

**MATURE T LYMPHOCYTE AND ACTIVATED T LYMPHOCYTE**

The mature T lymphocyte and activated T lymphocyte do not express the ALL antigen. Although the Ia-like antigen is not detected by standard cytotoxicity and indirect immunofluorescence techniques, a direct immunofluorescent assay has demonstrated that 1\%-8\% of normal peripheral blood T lymphocytes have this antigen,\textsuperscript{30} as do activated T lymphocytes.\textsuperscript{32}

The mature T lymphocyte unlike the T-ALL cell generally loses the ability to form E rosettes at 37\textdegree{}C but rosettes at 4\textdegree{}C and at room temperature.\textsuperscript{41} Numerous T-lymphocyte leukemias tested in our laboratory have identical surface membrane markers to those of a mature T lymphocyte. These leukemias include Sézary cell leukemia, T-chronic lymphocytic
leukemia (CLL), and T-lymphosarcoma cell leukemia.

PRE-B LYMPHOCYTE

Studies of lymphoid cells from the fetal livers of mice and humans have identified cells that have intracytoplasmic IgM without surface membrane immunoglobulin. The fact that they are synthesizing Ig suggests that they are B lymphocyte precursors (pre-B cells). Janossy and coworkers have identified a cell in normal human bone marrow whose phenotype is SmIg+, ClgM+, Ia+. The ALL counterpart to this cell is the pre-B cell ALL (subgroup X).

EARLY-B LYMPHOCYTES

As B lymphocytes mature they express surface membrane immunoglobulin and lose the ALL antigen. The ALL cell linked to this normal counterpart cell is found in subgroup XII. Subgroup XI has the ALL antigen and is most likely a "transition" cell between the pre-B cell and early-B cell.

MATURE B LYMPHOCYTE

The mature B lymphocyte has SmIg and Ia-like antigens. Most chronic B-lymphocyte leukemias such as CLL, hairy cell leukemia, and lymphosarcoma cell leukemia have surface markers identical to mature B lymphocytes.

PLASMA CELL

The plasma cell is the most differentiated B cell. Its surface membrane differs from the mature B lymphocyte in that it generally loses Ia-like antigens and SmIg. Its malignant counterpart is the myeloma cell that shares these properties. Like the mature B lymphocyte, the plasma cell does not express the ALL antigen.

CLINICAL CORRELATES

Approximately 90% of non-B, non-T-ALL patients in the pediatric population and 60% of the adult patients have both the Ia-like and ALL antigens (subgroup III). It has been reported that this group, at least in children, has a more favorable prognosis than the ALL patients without the "common ALL" antigen. This remains to be confirmed.

The T-ALL patients are generally older, male, often have a thymic mass and have a less favorable prognosis due to more rapid drug resistance than the non-B, non-T group. The six subgroups of T-ALL shown in this scheme generally fit this clinical picture. More important, 4 of the 6 patients with ALL cells that lacked receptors for sheep erythrocytes, but had T antigens, were teenage boys with thymic masses and high blast counts. Prior to identifying their cells as T antigen positive, these patients were inappropriately classified as non-B, non-T-ALL.

The B-ALL patients appear to be an unusual group that may be a leukemic phase of non-Hodgkin's lymphoma. These patients generally respond poorly to therapy. It is interesting to speculate that it is those ALL closest in origin to the stem cells (subgroups I–III) that have the most favorable prognosis, while those ALL cells that are more differentiated B and T cells have a less favorable prognosis.

Enzyme analysis of ALL cells may also contribute to an understanding of lymphoid differentiation. Terminal deoxynucleotidyl transferase (TdT), hexosaminidase isoenzyme patterns, adenosine deaminase and 5'-nucleotidase have all been useful in delineating subtypes of leukemic lymphoblasts. T lymphoblasts have been shown to differ from non-B, non-T lymphoblasts in that they have elevations in adenosine deaminase activity and decreased 5'-nucleotidase activity. In addition, a direct correlation has been reported between elevated levels of the first hexosaminidase component and the ALL-associated antigen.

Morphological classification has been of marginal value in classification and prediction of response to therapy in ALL. The French–American–British group has proposed three subtypes of ALL (L1, L2, and L3) based on morphological criteria. The rare L3 subtype appears to be a leukemic form of Burkitt's lymphoma, a B lymphocyte. However, the L1 and L2 subtypes, which represent >95% of ALL, are not differentiated immunologically, and their relation to the immunologic subgroups is not known.

There are certain limitations to the hypothesis concerning normal lymphoid differentiation based on antigenic classification of leukemic cells. First, malignant cells may have aberrant differentiation and may not, therefore, reflect the surface markers of normal cells. Our knowledge of more mature leukemias such as Sézary cell leukemia and chronic myelogenous leukemia suggest, however, that this phenomenon probably is not generally true. Second, some of the subgroups of ALL in our series comprise only one or two patients. Clearly more patients must be studied before one can draw firm conclusions about cellular antigenic patterns of the different subgroups. The purpose of this scheme was not to suggest an alternate classification of ALL, but to emphasize the marked heterogeneity of this disease, and its utility in understanding lymphoid maturation. Third, leukemic cell populations are sometimes heterogeneous, which might conflict with the hypothesis that there is a maturation arrest at a specific level of differentia-
tion. Our knowledge of leukemia suggests that it is often not a static process, but may evolve in time in a given patient. Evolution of membrane phenotypes and chromosomal abnormalities support the concept of a dynamic disease process. It might be anticipated, therefore, that at any given point in time, cells at different levels of differentiation might be present in the bone marrow and in the circulation. Does such heterogeneity pose a significant problem? We don't believe it does, and in fact it may strengthen the concept presented here. Greaves and coworker have reported that when testing for CIgM not all the cells from a patient with pre-B ALL were positive. This suggests that the target cell might not be the pre-B cell, rather a less mature cell that is capable of further differentiating to a pre-B cell, thus, only a portion of the cells are in fact pre-B cells. Certainly the same can be said for chronic myelogenous leukemia where the cells are in fact pre-B cells. Certainly the same can be said for chronic myelogenous leukemia where the target cell is a stem cell that is capable of further differentiation. Notwithstanding these limitations, we believe this conceptual framework that we propose for normal lymphoid differentiation is useful at this time. Additional information acquired in the future will undoubtedly lead to modifications of this scheme.

The role of cell surface marker antigen studies in the classification of ALL is limited by the variability of antisera used to define these antigens in different laboratories. Greater laboratory exchange of antisera and reference laboratories for standardization will be necessary before the full potential of immunologically defined classification can be appreciated. The development of monoclonal antibodies produced by the hybridoma technique of Kohler and Milstein is likely to provide the technology necessary for detailed and reproducible studies.

ACKNOWLEDGMENT

The authors wish to thank Drs. John Fitchen and Robert Ashman for review of the manuscript, and Bill Clark and Marilyn Makimoto for excellent technical assistance.

REFERENCES


Immunologic classification of acute lymphoblastic leukemia. Implications for normal lymphoid differentiation

KA Foon, RJ Billing, PI Terasaki and MJ Cline