Immunologic characterization of lymphocytic leukemias

Methodologic and immunophenotypic considerations

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■ Immunophenotyping has become an important adjunct to the routine morphologic and cytochemical evaluation of leukemias. The development and refinement of immunologic techniques applicable to the general clinical pathology laboratory and the commercial availability of lineage-specific monoclonal antibodies have enhanced this technology as a diagnostic tool. Lymphoid leukemias, in particular, can be accurately identified and subclassified according to their immunologic characteristics, as defined by lymphocyte differentiation antigens. Immunophenotypic classification has also been demonstrated to have prognostic significance for certain acute and chronic lymphoid leukemias. This review considers current immunotyping methods, characteristics of the antibody probes used, and the immunophenotypic characteristics of the acute and chronic lymphoid leukemias.

□INDEX TERMS: LEUKEMIA, LYMPHOCYTIC; LEUKEMIA, LYMPHOCYTIC, ACUTE □CLEVE CLIN J MED 1989; 56:722–739

LEUKEMIAS have traditionally been classified according to their cytomorphologic and cytochemical characteristics. In 1976, the French-American-British Cooperative Group (FAB)[1] established a system for the classification and nomenclature of acute myeloid and lymphoblastic leukemias, which has gained international recognition and acceptance. In contrast, the chronic lymphoid leukemias are not part of a unified classification system because they represent a heterogeneous group of disorders, each exhibiting unique clinicopathologic features, and therefore are considered independent entities. The concept of classifying lymphoid leukemias by morphologic and cytochemical criteria alone has recently been challenged by the rapidly expanding knowledge of lymphocyte ontogeny, as defined by immunologic markers. It is now possible to specifically subclassify lymphoid leukemias, not only as to whether they are of T-lymphocyte or B-lymphocyte origin, but also as to their level of maturational arrest, which corresponds with the normal stages of lymphocyte development within each subset.[2–5] Immunophenotyping has almost eliminated the undesirable category of undifferentiated acute leukemia because it is now possible to identify most of these cases as being of either myeloid or lymphoid lineage. Immunotyping has rapidly gained acceptance as an important adjunct to the routine diagnostic studies for leukemias. This reflects the development and refinement of immunologic techniques that can be applied in the general clinical pathology laboratory as well as the commercial availability of many useful monoclonal antibodies. This paper will review the methods currently used for im-

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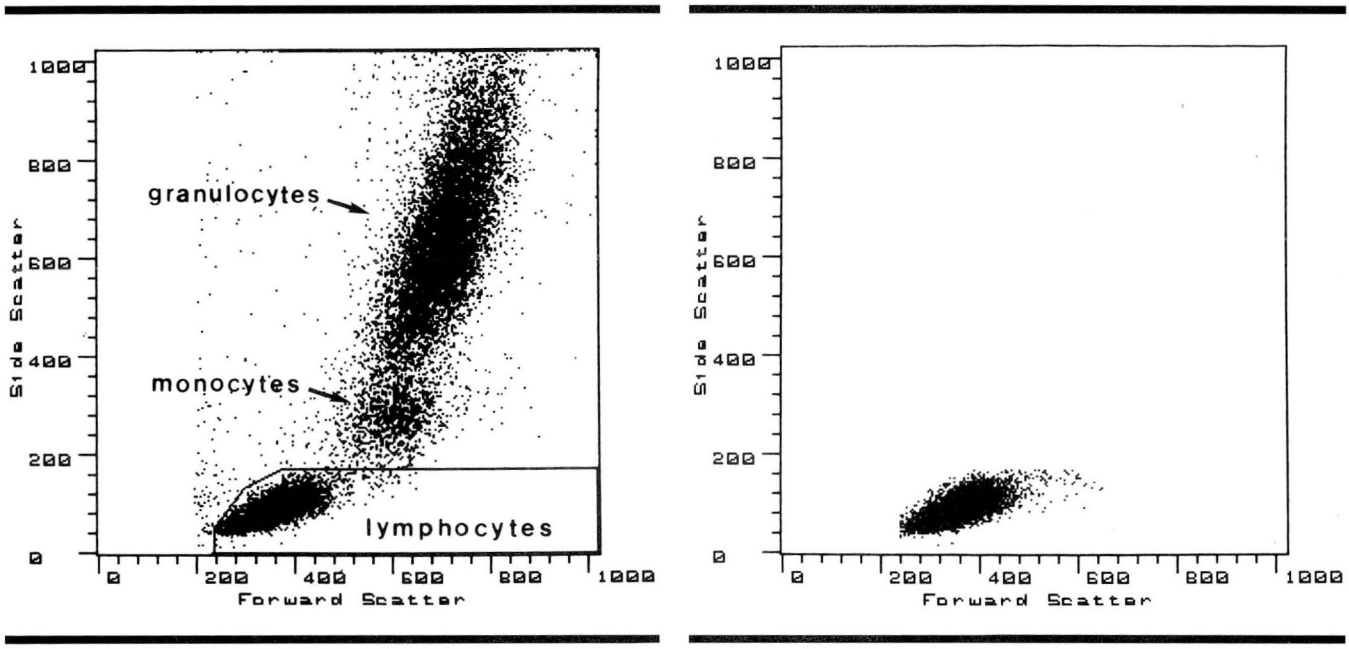

FIGURE 1A. Light-scatter histogram of whole blood with a "window" placed around the lymphocyte population for gating.
FIGURE 1B. Light-scatter histogram of whole blood after gating on the lymphocyte population.

munophenotyping, the antibody probes most frequently used, and the immunophenotypic characteristics of acute and chronic lymphoid leukemias.

METHODS FOR IMMUNOPHENOTYPING

The methods most frequently used to immunophenotype lymphoid leukemias include flow-cytometric analysis and the immunocytochemical evaluation of cytospin preparations, bone marrow smears, and peripheral blood smears.

Flow-cytometric analysis

Flow cytometry undoubtedly represents the most sensitive and efficient method available for immunophenotyping, but it is generally restricted to tertiary medical centers where large numbers of specimens and investigative work can justify and support the initial major capital expenditure and the laboratory operating expenses. This methodology may become common in clinical laboratories in the near future as smaller, less-expensive, and user-friendly instruments are developed and the clinical importance of accurate immunologic phenotyping becomes more widely recognized.

The sensitivity of the flow cytometer may be attributed to the laser source and the light-scatter/fluorescence detection system that provides multiparameter measurements of fluorochrome-labeled cells in suspension. Lasers with 4–5 W have traditionally been employed in most instruments; however, the current trend is to use low-power (15–25 mW), air-cooled argon-ion lasers (FACScan and Profile instruments) that are more sensitive, less expensive, and smaller (bench top). These instruments can detect approximately 500–1,000 fluorescent molecules/cell, far below the detection threshold of the human eye by manual fluorescent microscopy. A cytometer can also collect data on up to 1,000 cells/s with a high degree of precision and measure up to six parameters (cell size; cytoplasmic granulation; number of cells; and one, two, or three fluorescent labels). In addition, multiparameter data analysis can be performed using the instrument's computer system and analytic software. It is also possible to collect and selectively analyze specific subpopulations of cells with the cell-sorting capability of the more sophisticated instruments.

Peripheral blood or bone-marrow specimens collected in heparin or EDTA can be analyzed following the preparation of a mononuclear cell suspension by Ficoll-Hypaque density-gradient separation or the use of a whole-blood lysis technique.[6,7] The specimen should be processed as soon as possible, but certainly within six hours of collection to preserve cell viability and surface antigen density.[7] If this is not possible, tissue-culture

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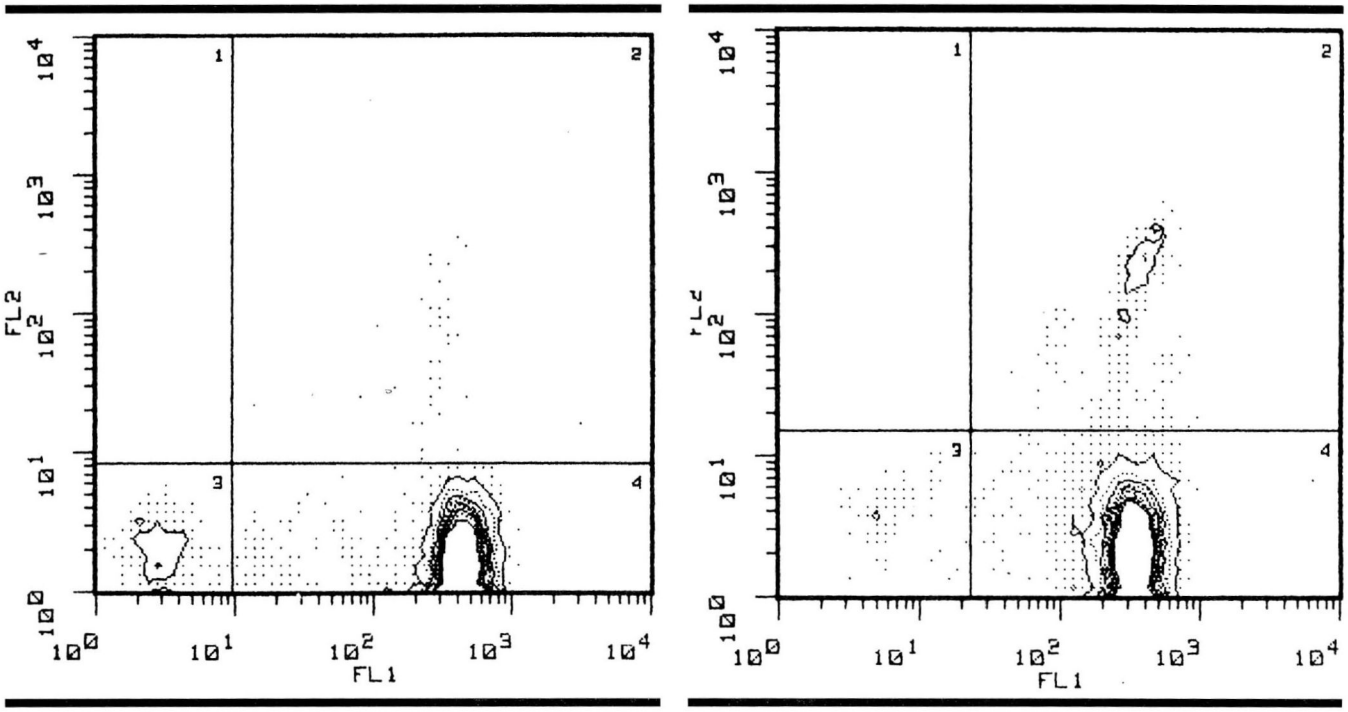

FIGURE 2. Two-color-flow cytometric histograms of two mononuclear cell suspensions from peripheral blood. FIGURE 2A. The lymphocytes are $CD45^+$ (FL1—green fluorescence) $CD14^-$ (FL2—red fluorescence) and are located in quadrant 4. Note the absence of monocytes ($CD45^+CD14^+$ cells) in quadrant 2. FIGURE 2B. A different specimen contains 4% monocytes in quadrant 2.

media (i.e., RPMI 1640 with L-glutamine [Gibco Laboratories, Boston]) should be added to the whole-blood specimen at a sample-to-medium ratio of 1:2 or 1:4,[6] thus allowing delay of cell separation for 24–48 hours.

Ideally, the mononuclear cell suspension should contain approximately 10–20 × 10^6 cells/mL, although a lower concentration is acceptable, depending on the number of antibodies in the profile. Efficient analysis requires 0.25–1.0 × 10^6 cells per tube for either single- or two-color analysis. The viability of the cells in suspension, as determined by a supravital stain such as trypan blue, should be no less than 90%. It may be necessary at times to accept a less viable specimen because of the cellular necrosis that accompanies some acute leukemias. The data must then be interpreted with caution because of nonspecific antibody binding (false positives) and nonreactive cells (false negatives). Also, if the suspension is prepared more than one hour after specimen collection, monocytes and granulocytes will be more likely to contaminate the preparation.[6] Therefore, it is also beneficial to morphologically examine a Wright's-stained cytospin preparation from the suspension and obtain a differential cell count to ascertain the purity of the preparation. This becomes important when one considers that some monoclonal antibodies, presumed to be lymphocyte specific, are found to react with monocytes and/or granulocytes.

The whole-blood lysis method uses ammonium chloride or a commercial red-cell lysing agent (Immuno-Lyse, Coulter Immunology, Hialeah, Fla., and FACS Lysing Solution, Becton Dickinson Monoclonal Center, Mountain View, Calif.) after the cells are stained. A differential loss of some white cells and cell death may result if there is prolonged exposure to the lysing agent (>5 minutes). This method significantly reduces processing time but requires careful selection of the cell population to be analyzed by the flow-cytometry operator since large numbers of granulocytes and monocytes are present in the cell suspension. This is accomplished for both the mononuclear cell suspension and whole-blood specimens by electronic "gating," in which a certain population of cells (lymphocytes or blasts) are selected for fluorescence analysis based on their forward (low-angle) and 90° (side-angle) light-scatter characteristics (*Figure 1A* and *1B*). Monocytes should consti-

tute less than 5% of the population being analyzed after gating, and this may be checked using two-color analysis with the monoclonal antibodies CD45 and CD14 (Simultest Leuco-GATE, Becton-Dickinson, and Cyto-Stat, Coulter Clone). Cells coexpressing CD45 and CD14 represent monocytes (*Figure 2A* and *2B*).

Regardless of which specimen-preparation technique (Ficoll-Hypaque separation or whole-blood lysis) is used, the detection of cell antigens may proceed using either direct or indirect staining techniques.[7] These methods are best suited for the detection of high-density and low-density antigens, respectively. Prior to antibody labeling, the cells should be maintained at room temperature to avoid decreased antigen resolution (e.g., Leu-8$^+$ cells) and the loss of certain leukocyte populations (T lymphocytes),[6] which can occur at 4° C. After labeling, the cell suspensions must be held at 4° C to prevent capping and internalization of the antigen-antibody complex. These phenomena will be discussed in more detail. It may also be desirable, depending upon the experience of the flow-cytometry operator, to examine the labeled cell suspensions by immunofluorescence microscopy to evaluate the quality of the immunofluorescence staining. If analysis is to be delayed longer than one to two hours, fixation in 2% paraformaldehyde is recommended. However, fixation of more than two hours can decrease the staining intensity of the Leu-15 antigen.

Although flow cytometry represents the method of choice for immunophenotyping cell suspensions, its value depends directly on high-quality specimen preparation, an experienced flow-cytometry operator, experience with the specificity of antibodies used, and experience in the interpretation of flow-cytometric histograms. Several reviews provide more detailed information concerning flow-cytometric analysis and sample preparation.[6–10]

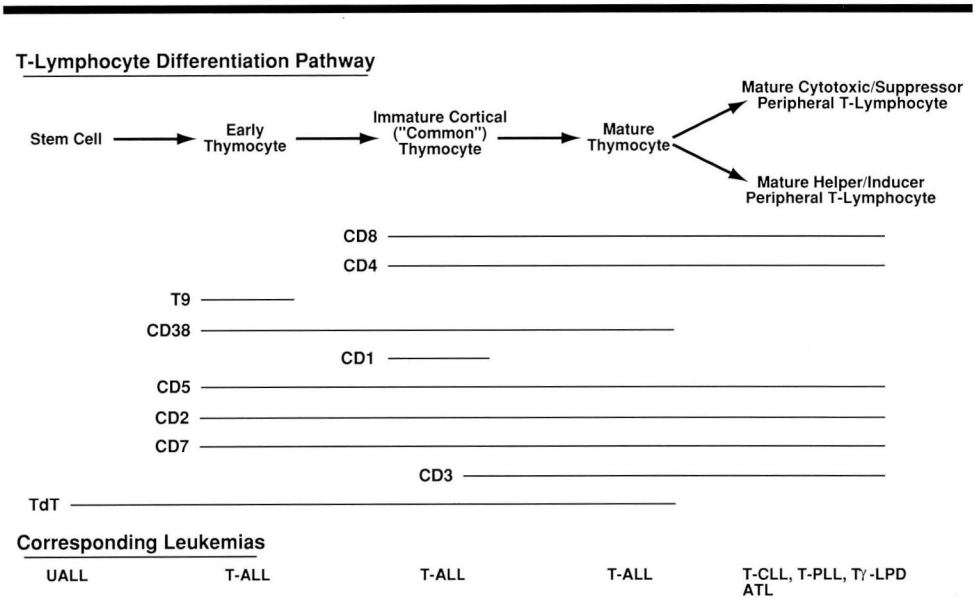

FIGURE 3. Stages of T-lymphocyte differentiation with associated antigens and corresponding leukemias. CD7 is present on some cortical and medullary thymocytes, most peripheral T cells, and >95% of T-ALL. CD4 and CD8 are coexpressed in common thymocytes but are independently expressed in mature thymocytes and peripheral T lymphocytes. *ALL* = acute lymphoblastic leukemia, *T-ALL* = T-cell ALL, *ATL* = adult T-cell leukemia/lymphoma, *T-CLL* = T-cell chronic lymphocytic leukemia, *T-PLL* = T-cell prolymphocytic leukemia, *Tγ-LPD* = Tγ-lymphoproliferative disease, and *UALL* = undifferentiated ALL.

Immunocytochemical evaluation of cytospin preparation

An alternative to flow cytometry, and sometimes complementary to it, is the immunocytochemical evaluation of cytospin preparations. Briefly, a mononuclear cell suspension is prepared by Ficoll-Hypaque separation of bone marrow or peripheral blood, and the cell count of the cell suspension is adjusted so that an uncrowded monolayer of cells is obtained upon cytocentrifugation. Multiple slides are prepared (air-dried) for staining, and unused slides may be stored at room temperature for up to one month without significant loss of antigenic reactivity. Following acetone fixation, the slides are immunostained using an avidin-biotinylated peroxidase complex technique.[11] The immunoreactivity of the cells is then evaluated using light microscopy. This relatively simple methodology has been demonstrated to correlate closely with flow-cytometric analysis[12] and offers the advantages of concurrent correlation of cytomorphologic features with the surface-marker staining pattern, identification of contaminating cells, and the convenience of a permanent record.

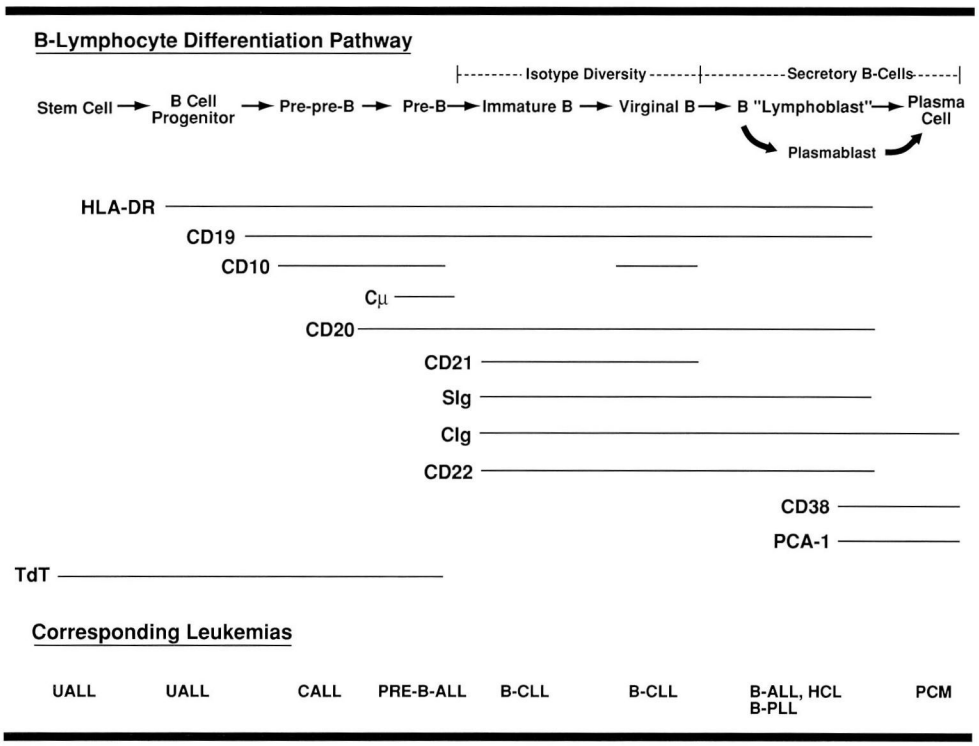

FIGURE 4. Stages of B-lymphocyte differentiation with associated antigens and corresponding leukemias. Although CD10 is expressed during normal B-cell ontogeny at the virginal B stage, it is only rarely observed in CLL. *ALL* = acute lymphoblastic leukemia, *CALL* = common ALL, *B-PLL* = B-cell prolymphocytic leukemia, *HCL* = hairy-cell leukemia, *PCM* = plasma-cell myeloma, *SIg* = surface immunoglobulin, *CIg* = cytoplasmic immunoglobulin, Cμ = cytoplasmic IgM heavy chain.

Immunocytochemical evaluation of bone-marrow smears and imprints

Occasionally, cases will be encountered in which an unsuspected leukemia is discovered on examination of bone-marrow smears, and no additional specimen can be obtained. In this situation, unstained smears and/or bone-marrow biopsy imprints may be immunostained using special techniques that block or avoid the unwanted endogenous peroxidase activity present in erythrocytes and myeloid elements. Methanolic-peroxide blocking of endogenous peroxidase, commonly used for routine immunoperoxidase staining, usually results in the loss of surface antigen reactivity. Optionally, a glucose-oxidase block can be used that has been shown to completely inhibit endogenous peroxidase activity without having a deleterious effect on surface antigens.[13] It is essential that whenever a blocking agent is used, appropriate parallel positive controls be run to ensure preservation of antigenicity.

An attractive alternative to the immunoperoxidase method is the use of immunoalkaline phosphatase[14] or alkaline phosphatase anti-alkaline phosphatase (APAP) immunocytochemical techniques.[15] These methods are said to exhibit sensitivity and advantages similar to the immunoperoxidase technique,[16,17] and although endogenous leukocyte and monocyte alkaline phosphatase activity can cause background staining, they are effectively blocked by adding levamisole and tartrate to the alkaline phosphatase substrate medium.[18,19]

Glucose oxidase anti-glucose oxidase is another enzyme-labeled method, which has been advocated as the best alternative to immunoperoxidase or immunoalkaline phosphatase because mammalian cells lack this enzyme.[20] However, this method has been found to be significantly less sensitive than the others, requiring prolonged incubation times for optimal staining.[19]

ANTIBODY PROBES FOR IMMUNOPHENOTYPING

The immunophenotyping of lymphocytes is based on the use of monoclonal and polyclonal antibodies (surface markers) to identify lymphocyte differentiation antigens and cytoplasmic and surface immunoglobulin (CIg and SIg, respectively). Using these markers, it has been possible to immunologically define the differentiation pathways for both T lymphocytes and B lymphocytes (*Figures 3* and *4*). This has provided the foundation for the immunologic subclassification of the lymphoid leukemias and supported the theory that lymphoid neoplasms develop as a result of the emergence of a neoplastic clone of lymphoid cells that exhibit an arrest of maturation, which corresponds to a particular stage of lymphocyte ontogenesis.[21,22]

TABLE 1
SURFACE MARKERS COMMONLY USED IN IMMUNOPHENOTYPING LYMPHOID LEUKEMIAS

Antigen/antibody designation	Antigen cluster designation*	Cellular expression
T-lymphocyte markers		
T1, Leu-1, T101	CD5	Thymic and peripheral T cells, B-CLL cells
T3, Leu-4	CD3	Peripheral T cells
T11, Leu-5	CD2	Thymic and peripheral T cells
T10, Leu-17	CD38	Immature thymocytes, activated T cells, plasma cells
T9	CD71	Early thymocytes (Stage I), activated lymphocytes, monocytes, all cells having transferrin receptors
T6, Leu-6	CD1	Common thymocytes (Stage II), Langerhan's cells
T4, Leu-3	CD4	Helper-inducer T cells
T8, T5, Leu-2	CD8	Cytotoxic-suppressor T cells
Leu-9, 3A1	CD7	Peripheral T cells, some cortical and medullary thymocytes, most T-ALL cells
Leu-7, HNK-1	CD57	Subsets of cytotoxic suppressor T cells and NK cells
Leu-11	CD16	NK cells, neutrophils
Tac, IL-2	CD25	Activated T cells, NK cells, HTLV-infected T-cell leukemia lines
B-lymphocyte markers		
B1, Leu-16	CD20	Pre-B, immature, virginal, and secretory blast-stage B cells
B2, CR11	CD21	Immature and virginal B cells
B4, Leu-12	CD19	Pre-pre-B, pre-B, immature, virginal, and secretory blast-stage B cells
Leu-14	CD22	Some pre-B, immature, virginal and secretory blast-stage B cells, and hairy-cell leukemia
BA-1	CD24	Pre-pre-B, pre-B, immature, virginal and secretory blast-stage B cells
CALLA, J5, BA-3	CD10	Pre-pre-B, pre-B, some virginal B cells, CALL cells
PCA-1	–	Plasma cells (late secretory B cells)
Other markers		
HLA-DR, OKIa, I2	–	B cells, activated T cells, monocytes/macrophages, Langerhan's cells
Mo1, OKM1, Leu-15	CD11b	Monocytes, granulocytes, NK cells, suppressor T cells
Leu 8, TQ1	–	T cells, B cells, neutrophils, monocytes
Leu-M3	CD14	Monocytes/macrophages
Leu-M5	CD11c	Monocytes, macrophages, acute myeloid leukemias, hairy-cell leukemia
LCA, T200, HLe-1	CD45	Leukocytes

*The antigen cluster designation is now the preferred terminology for the leukocyte differentiation antigens.
B-CLL = B-cell chronic lymphocytic leukemia, *NK* = natural killer, *B-ALL* = B-cell acute lymphoblastic leukemia, and *CALL* = common acute lymphoblastic leukemia

Leukocyte differentiation antigens

Most antigens that phenotypically distinguish cellular differentiation are located on the cell surface and represent functional molecules, some of which are receptors involved with specific cellular activities. A vast array of commercially available monoclonal antibodies have been developed for these antigens by a growing number of manufacturers. Consequently, there is considerable duplication of products with variable sensitivity, purity, and epitope specificity. Until recently, there was no uniform nomenclature for antigen designation, causing significant confusion among users because at least three systems were used (Coulter, Becton Dickinson, and Ortho). However, a standard nomenclature was adopted by the Second International Workshop on Human Leukocyte Differentiation Antigens, termed clusters of differentiation or cluster designations (CD).[23,24] The CD is now the preferred terminology for leukocyte differentiation antigens. The antibodies most

TABLE 2
LYMPHOCYTE DIFFERENTIATION ANTIGENS ASSOCIATED WITH SPECIFIC CELLULAR FUNCTIONAL ACTIVITIES OR SURFACE RECEPTORS

Antigen	Associated functional activity or surface receptor
CD3	Recognizes MHC restricting elements on macrophages
CD4	T-cell mediated helper-induced functions
CD8	T-cell mediated cytotoxic-suppressor functions
CD2	Sheep erythrocyte rosette receptor
CD16	NK activity and the Fc IgG receptor of NK cells and neutrophils
CD71	Transferrin receptor
CD38	Activated T cells
CD21	C3d receptor and EBV receptor
CD25	Interleukin-2 receptor (T-cell growth factor receptor) and activated T cells
CD11b	CR_3 receptor (C3bi receptor) and T-cell-mediated suppressor functions
HLA-DR	Activated T cells

MHC = major histocompatibility complex, *NK* = natural killer, *EBV* = Epstein-Barr virus, and *C3bi* = complement component.

TABLE 3
LEUKOCYTE DIFFERENTIATION ANTIGENS EXHIBITING NONLINEAGE RESTRICTED EXPRESSION

Antigen	Expected cellular expression	Nonlineage restricted cellular expression
CD5	T cells	B chronic lymphocytic leukemia
CD4	T cells	Monocytes/macrophages*†
CD1	Thymocytes	Langerhan's cells
CD38	Thymocytes	Plasma cells, activated T cells, NK cells, B-cell subset, lymphoid and myeloid leukemias
CD7	T cells	Monocytes,* occasional cases of acute nonlymphocytic leukemia, NK cells
CD16	NK cells	Neutrophils
CD19	B cells	Monocytes*
CD21	B cells	Monocytes and granulocytes*
CD10	Pre-B cells	T acute lymphoblastic leukemias (40%), neutrophils
CD11c	Monocytes/macrophages	Granulocytes,* large granular lymphocytes,* hairy-cell leukemia

*Low-density surface antigen or weak antibody reactivity
†Cytoplasmic as well as surface antigen reactivity

useful in phenotyping lymphoid leukemias and those associated with specific functional activities or surface receptors are shown in *Tables 1* and *2* with their commercial designation and antigen CD.

The specificity of monoclonal antibodies derives from the fact that each recognizes a single epitope, or portion of an antigen, which is composed of only a few amino acids.[25] However, the entire antigen may contain thousands of amino acids,[26] and therefore, it is possible for multiple monoclonal antibodies to have the same antigen specificity but recognize different epitopes within the antigen structure. This accounts for some of the variability occasionally observed in the sensitivity and specificity of monoclonal antibodies developed against the same antigen by different manufacturers. An excellent example of this problem is the recently recognized epitope deficiency noted in a significant portion of the black population as recognized by the OKT4 monoclonal antibody (Ortho) but not observed when using similar clones anti-T4 (Coulter) or anti-Leu-3a (Becton Dickinson).[27]

"Lymphocyte-specific" monoclonal reagents may also exhibit cross-reactivity with similar antigens on other cell types. This "non-lineage-restricted" antigen expression can cause errors and considerable difficulty in the interpretation of flow-cytometric data and immunostained cytologic preparations, especially when there is poor preservation of cytomorphology in the latter. This problem can be further complicated because other cell types (monocytes and occasionally granulocytes) known to nonspecifically bind monoclonal antibodies may be found in the mononuclear layer of Ficoll-prepared specimens; therefore, familiarity with the specificity of the antibodies being used and their potential for cross-reactivity with other cellular elements is essential. Leukocyte-differentiation antigens having a non-lineage-restricted distribution are shown in *Table 3*.

Activated T lymphocytes, B lymphocytes, natural killer cells, and phagocytic cells all possess Fc receptors to which whole antibodies may bind.[28] This could obviously result in the misidentification of cell populations, regardless of which analysis method is used. This problem can be substantially alleviated by using the $F(ab')_2$ fragment of antibodies or by incubating the cell suspension at 37° C for 15–45 minutes with heat-inactivated pooled human AB serum prior to adding the primary antibody.[6,29] Since monocytes/macrophages have high-density Fc receptors, it may be desirable to remove them from the cell suspension with carbonyl ion particles if they are present in high concentration.[12] Flow-cytometric analysis can further circumvent many of the problems that contaminating cell populations (granulocytes and monocytes) cause by carefully gating them out as previously described.

Another interesting and well-recognized phenomenon is the deletion or weak expression of surface antigens associated with lymphoid neoplasms. Normal lymphocytes exhibit a predictable array of differentiation antigens that reflect their stage of development. Neoplastic lymphocytes, on the other hand, not infrequently demonstrate the deletion of one or more of these antigens; this is observed more commonly in T-cell neoplasms. For this reason, it is recommended that the surface marker profile include monoclonal antibodies to more than one pan-T cell or pan-B cell differentiation antigen (e.g., CD5, CD7, CD2, CD20, CD19, CD24). This may be particularly valuable in determining whether a T-cell proliferation is benign or malignant since surface antigen deletions are presumptive evidence of neoplasia.

Surface and cytoplasmic immunoglobulins

The identification of immunoglobulin (Ig) synthesis is accepted as the most definitive marker of B-cell lineage. SIgs and CIgs are present in developing lymphocytes and correspond with specific stages of differentiation. Cytoplasmic mu heavy chains (Cμ) without light chains are the first Ig component produced during B-cell ontogenesis and are found in pre-B cells. These cells subsequently begin synthesizing light chains and the whole immunoglobulin M (IgM) molecule is then found in the cytoplasm and cytoplasmic membrane (SIgM), thus becoming functional B lymphocytes. Coexpression of surface immunoglobulin D (SIgD) is also observed at this stage, with the switch to additional isotypes (IgA, IgG, and IgE) occurring somewhat later in development. SIg functions as the receptor for antigen. When antigen is recognized, there is clonal expansion of the B lymphocytes, some of which become terminally differentiated plasma cells producing "secretory Ig." This secretory form of Ig is structurally different from its predecessor and is found in the cytoplasm but is poorly incorporated into the SIgM. Therefore, plasma cells express CIg but not SIg (*Figure 4*).[30]

The finding of light-chain restriction in a B-cell proliferation remains the most reliable indicator of neoplasia. Both heavy and light chains can be detected using flow-cytometric analysis or immunocytochemical staining of cytologic preparations. Immunostaining is usually accomplished using a direct method with a fluorescein- or peroxidase-conjugated antibody (preferably $F(ab')_2$ fragments) to human Ig heavy and light chains. The methodology is relatively simple, but there are certain caveats associated with its use.

The first is the nonspecific staining that may result from cytophilic Ig binding to Fc receptors. This is usually IgG and can be eliminated by including a one-hour incubation of the lymphocytes at 37° C in the absence of human Ig. It is also possible to remove the SIg using pronase and, following an overnight incubation, examine the cells for regeneration of SIg. This technique may be most valuable when the patient is known to have a serum M component.[31]

Nonspecific immunostaining is frequently due to dead or damaged cells, and these exhibit a heterogeneous staining pattern. Malignant cells are usually more fragile than normal cells, especially those with high mitotic activity, and thus a higher percentage of nonviable cells may be expected. It is therefore imperative that specimens be processed expeditiously and that viability testing be performed on all specimens for flow-cytometric analysis. This is less of a problem when immunostaining cytospin preparations because the necrotic or degenerating cells are usually easily identified.

As mentioned earlier, it is necessary to maintain the cell suspension at 4° C during staining to avoid the loss of SIg and/or the induction of an abnormal Ig distribution on the cell surface that could interfere with flow-cytometric analysis and the subsequent interpretation of data. Two patterns may be observed. The first, known as "patching," occurs after the SIg is cross-linked by anti-Ig, causing a clumped distribution due to the mobility of SIg in the lipid membrane. Although this will occur regardless of the reaction temperature, the second phenomenon, "capping," can be avoided. This reflects the movement of SIg to one pole of the cell and will occur if any metabolic activity is present. Occurring simultaneously with capping is the loss of SIg-anti-Ig complexes from the cell surface, termed "shedding."[31,32]

The importance of avoiding the binding of whole antibodies to the Fc receptors of nontarget cells has been discussed. However, it cannot be overemphasized that $F(ab')_2$ fragments are the reagents of choice when attempting to identify SIg or CIg. Polyclonal antibodies to heavy chains and kappa and lambda light chains are readily available in this form. Unfortunately, most murine monoclonal antibodies do not withstand the enzymatic digestion required to produce these fragments and are thus difficult to obtain.[29]

Although SIg can be easily detected by light or immunofluorescence microscopy, identification of Cμ is frequently difficult. This reflects the technical difficulty in distinguishing between true CIg and SIg present on the SIgM. The standard method for identifying Cμ compares the direct immunofluorescence staining of fixed with unfixed blood or bone-marrow lymphoblasts.[33] The fixed cells permit the anti-Ig antibody to enter the cell and react with any CIg present. Therefore, fixed cells will demonstrate Cμ, CIg, or CIg and SIg, whereas only SIg will be observed on the unfixed cells.

IMMUNOPHENOTYPIC CHARACTERIZATION OF LYMPHOID LEUKEMIAS

Acute lymphoblastic leukemia

The lymphoblastic leukemias have been well defined morphologically and are subclassified into three groups (L1, L2, and L3).[1,34] This classification system is of limited value in predicting the biologic behavior of the disease, although it has been shown that the prognosis with the L1 subtype is better in children and that the survival is uniformly poor with the L3 subtype, regard-

TABLE 4
IMMUNOPHENOTYPIC CLASSIFICATION OF NON-T ACUTE LYMPHOBLASTIC LEUKEMIA

Stage of differentiation	Differentiation antigens							Phenotype frequency (%)
	Ia	CD19	CD10	CD20	Cμ	SIg	TdT	
Early B-ALL								70–80
Stage I	+	–	–	–	–	–	+	4
Stage II	+	+	–	–	–	–	+	14
Stage III	+	+	+	–	–	–	+	33
Stage IV	+	+	+	+	+/–	–	+	49
B-ALL	+	+	+/–	+	–	+	–	<5

Note: Stages I–III may be considered pre-pre-B ALL.
Approximately 50% of Stage IV ALL display Cμ (pre-B).
CD10 is usually expressed in B-ALL, but it may be absent.

TABLE 5
IMMUNOPHENOTYPIC CLASSIFICATION OF T-CELL ACUTE LYMPHOBLASTIC LEUKEMIA

Stage of differentiation	Differentiation antigens										Phenotype frequency (%)	
	CD7*	CD5	CD2	CD38	CD71	CD1	CD3	CD4†	CD8†	TdT	Reinherz et al[5]	Roper et al[50]
Early thymocyte (I)	+	+	+	+	+	–	–	–	–	+	71	33
Common thymocyte (II)	+	+	+	+	–	+	+/–	+	+	+	24	37
Mature thymocyte (III)	+	+	+	+	–	–	+	+/–	+/–	+	5	30

*CD7 is present is >95% of T-ALL.
†CD4 and CD8 are independently expressed at the mature thymocyte stage of development.
Four cases in this study could not be categorized and appeared to represent transition forms between stages.

less of age.[34–36] In adults, it is unclear whether the L1 morphology has a more favorable prognosis than L2 when evaluated as an independent variable. The immunologic characterization of acute lymphoblastic leukemia (ALL), on the other hand, has defined prognostically significant subgroups not previously recognized and advanced understanding of this disease and its relationship to normal lymphocyte ontogeny.[3–5]

L3-ALL, also known as Burkitts' leukemia, is a B-cell neoplasm. It represents less than 5% of all lymphoblastic leukemias and is considered to be a bone-marrow manifestation of small, noncleaved cell-malignant lymphoma.[37] This is the most aggressive form of ALL, having a consistently poor prognosis. The leukemic cells usually demonstrate monoclonal CIg and SIg, which is frequently IgM, although cases lacking SIg have been reported.[38,39] The B-cell-associated antigens that are typically expressed include Ia, CD19, CD10, and CD20. TdT is consistently absent, in contrast to the other lymphoblastic leukemias in which it is present.[40]

The L1 and L2 subtypes constitute a heterogeneous group of T-cell and non-T-cell leukemias. The latter were formally designated "null-cell" acute lymphoblastic leukemias, but it is now known that these cases were of early B-cell lineage, accounting for approximately 70%–80% of all ALL. Nadler et al[4] recently proposed that, immunophenotypically, these represent early stages of B-cell differentiation, and they have subdivided this group based upon the expression of four B-cell-associated antigens (Ia, CD19, CD10, and CD20). The four subgroups include Ia^+ $CD19^-$ $CD10^-$ $CD20^-$ (4%), Ia^+ $CD19^+$ $CD10^-$ $CD20^-$ (14%), Ia^+ $CD19^+$ $CD10^+$ $CD20^-$ (33%), and Ia^+ $CD19^+$ $CD10^+$ $CD20^+$ (49%). The latter group also contained $C\mu^+$ (pre-B-ALL) and $C\mu^-$ cases. The CD19 antigen was found to be present in >95% of non-T-ALL and absent in T-ALL, indicating that it is a very reliable marker of B-cell lineage. The clinical significance of these phenotypes is unclear, although it was observed that most non-T-ALL cases in children less than two years old were Ia^+ $CD19^+$ $CD10^-$ $CD20^-$ while the majority of adult non-T-ALL cases were Ia^+ $CD19^+$ $CD10^+$ $CD20^+$. The presence of CD10 antigen indicates a more favorable prognosis,[41–45] whereas the pre-B phenotype, defined by the presence of Cμ, [33,46,47] has a

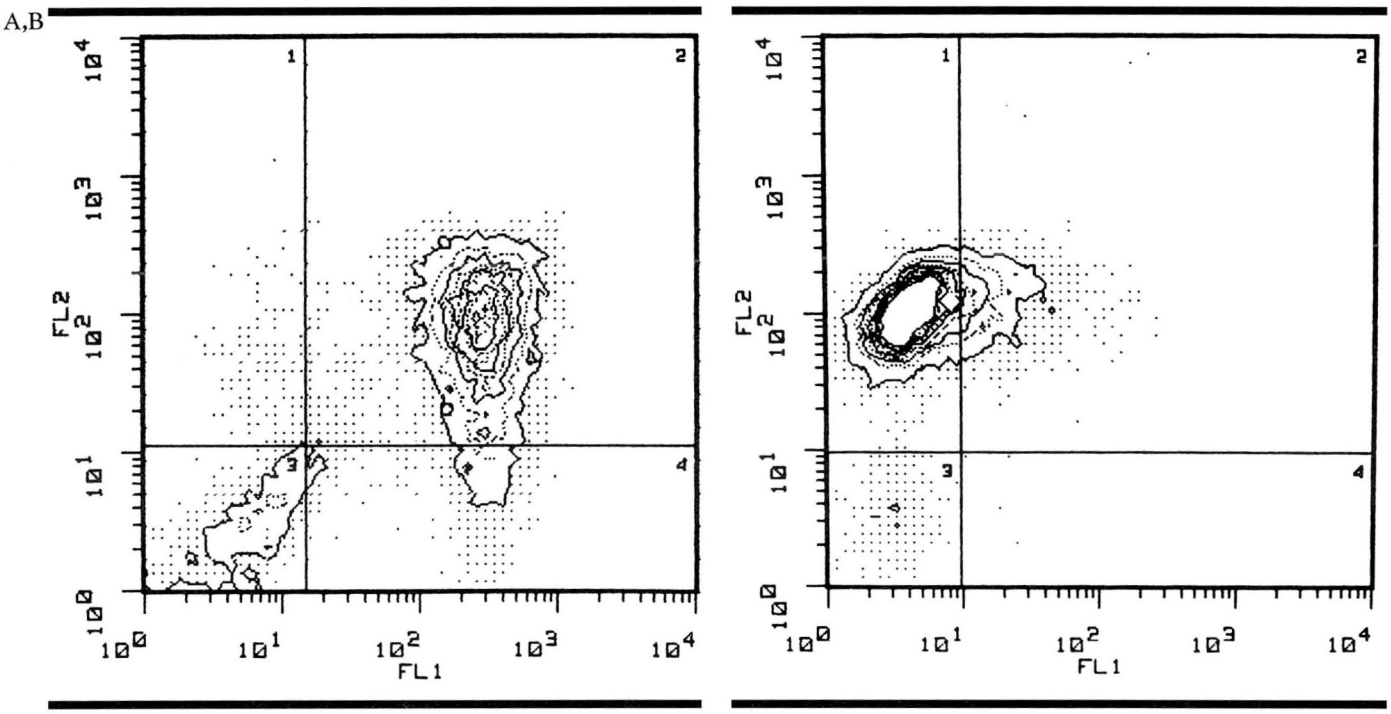

FIGURE 5. Two-color flow-cytometric histograms of an early B-cell acute lymphoblastic leukemia from peripheral blood. FIGURE 5A. Coexpression of CD10 (FL1) and HLA-DR (FL2) by the lymphoblasts in quadrant 2. FIGURE 5B. Most of the leukemia cells express CD19 (FL2) with only partial coexpression of CD20 (FL1)in quadrant 2.

somewhat less favorable prognosis.[48–50]

The T-ALL represents 15%–20% of all lymphoblastic leukemias and has a relatively poor prognosis. It has been immunophenotypically subclassified into three distinct groups, corresponding to the proposed developmental stages of thymic lymphocytes: early thymocyte (stage I) ($CD38^+$ and $CD71^+$), common thymocyte (stage II) ($CD38^+$, $CD1^+$, $CD4^+$, and $CD8^+$), and the mature thymocyte (stage III) ($CD38^+$, $CD5^+$, $CD3^+$, and $CD4^+$ or $CD8^+$).[51] However, this developmental scheme may be incomplete, as evidenced by recent studies by Blue et al[52,53] and Link et al.[54] The latter described the cytoplasmic expression of CD3 in most T-ALL cases, even those lacking surface CD3. In addition, CD7 appears to be expressed in almost all cases,[55] as is TdT, but other pan-T-cell antigens (CD2 and CD5) may be deleted. Remarkably, CD10 has also recently been found in 42% of T-ALL.[56] Controversy exists regarding the phenotypic distribution of cases,[51,57] however, this distinction is not critical since no prognostic relevance has been attributed to any of the stages of differentiation.

Two recent publications extensively reviewed the application of monoclonal antibodies and the use of flow cytometry in the diagnosis of acute leukemia.[58,59] *Tables 4* and *5* summarize the immunophenotypic characteristics of the ALLs, and *Figure 5* demonstrates two-color flow-cytometric histograms of an early B-ALL.

Adult T-cell leukemia-lymphoma

Adult T-cell leukemia-lymphoma (ALL) is a unique clinicopathologic entity that was first described by Uchiyama et al[60] in 1977 and appeared to be geographically restricted to the southernmost islands of Japan. However, additional cases of ALL have subsequently been found to be clustered in the Caribbean basin[61] and southeastern United States[62] with sporadic cases reported outside these areas.[63,64] The most significant feature of ALL is that it is a "sentinel disease" for human T-cell leukemia-lymphoma virus (HTLV-1) infection; most afflicted patients have antibody to this virus.[65,66] ATL is an aggressive disease characterized by adult onset, generalized lymphadenopathy and hepatosplenomegaly, frequent skin involvement, no mediastinal involvement, hypercalcemia, and pleomorphic leukemic T cells with lobulated nuclei in bone marrow and peripheral blood. The leukemic cells usually demon-

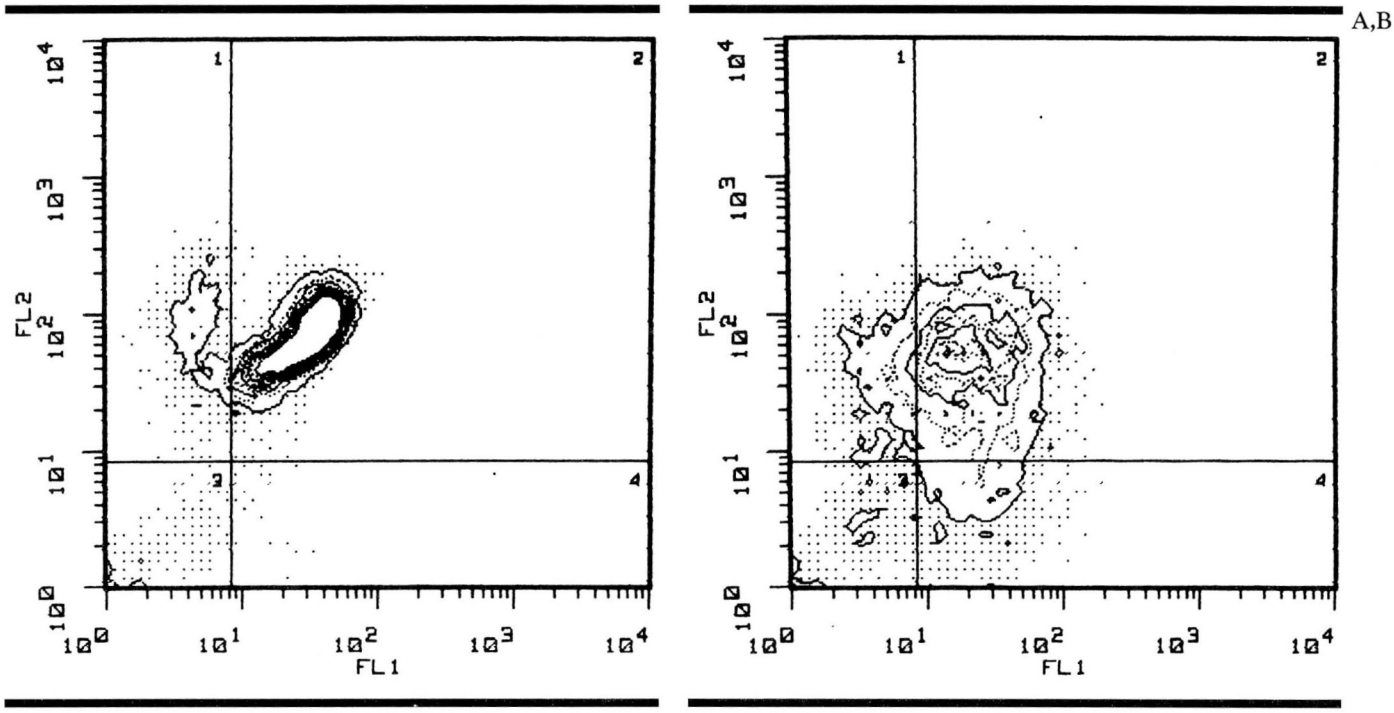

FIGURE 6. Two color flow-cytometric histograms of CLL from peripheral blood. FIGURE 6A. The leukemic cells coexpress CD19 (FL1) and CD5 (FL2) in quadrant 2, while CD5+ non-neoplastic T lymphocytes are segregated into quadrant 1. FIGURE 6B. Neoplastic lymphocytes coexpressing CD20 (FL1) and Leu-8 (FL2).

strate a mature T-cell phenotype of the helper-inducer subtype ($CD5^+$, $CD3^+$, and $CD4^+$)[67,68] and express the surface receptor for T-cell growth factor (interleukin-2), as recognized by anti-TAC (CD25).[69,70] Many also exhibit suppressor functional activity despite a helper phenotype.[68] Other phenotypes (Ia^+, $CD2^+$, $CD7^+$, $CD4^-$, and $CD2^+$, $CD3^+$, $CD4^+$, $CD8^+$, Ia^+) have been described,[63,64] and TdT activity has been reported in four cases.[63,71]

Chronic lymphocytic leukemia

Chronic lymphocytic leukemia (CLL) is a neoplastic proliferation of small lymphocytes that involve the bone marrow, peripheral blood, and lymph nodes to a variable extent. They are usually indistinguishable from normal small lymphocytes but may demonstrate morphologic heterogeneity, making the distinction from a circulating lymphoma difficult at times.[72] Approximately 95% of the cases are of B-cell origin and have high peripheral-blood lymphocyte counts, splenomegaly, lymphadenopathy, and a chronic clinical course. However, early- or low-grade forms of this disease, characterized by chronic mild lymphocytosis, are now being recognized through the use of flow-cytometric analysis.[73–75] The B lymphocytes express many B-cell antigens (Ia, CD20, CD19, CD21, and CD24), and rare $CD10^+$ cases have been reported.[76] They have low-density SIg (compared to normal lymphocytes), and this is usually IgM or IgM and IgD with kappa or lambda light-chain restriction.[77,78] A significant portion of these cells have receptors for mouse erythrocytes (MRBC) and will form MRBC rosettes. This attribute may be helpful in distinguishing this entity from other lymphoproliferative disorders.[79] An unusual characteristic of B-CLL is the presence of a T-cell-restricted surface antigen, CD5.[80] The unique combination of Ig light-chain monoclonality and the coexpression of CD5 and CD19 surface antigens in a morphologically mature population of lymphocytes is consistent with B-CLL (*Figure* 6A).[75] However, the coexpression of these markers has also been observed in B-cell lymphomas, the small lymphocytic (WDLL) and intermediate lymphocytic types, necessitating the inclusion of circulating lymphoma cells in the differential diagnosis.[81–83] It has been demonstrated, though, that lymphocytic lymphomas have high-density SIg,[84] and this may be useful in separating these two entities.

Attempts have been made to subclassify B-CLL immunologically to identify clinically significant phenotypes. An immunophenotypic subclassification has been proposed that defines specific stages of lymphocyte maturation arrest, but there was no apparent correlation with the stage of the disease.[85] Other studies have correlated the lymphocyte SIg isotype with survival and stage of disease at presentation with conflicting results.[86,87] Recently, a common variant of B-CLL was described in which 80% of the cases studied displayed another T-cell antigen, TQ1 (Leu-8) (*Figure 6B*), which in normal T-helper lymphocytes defines a subset responsible for inducing suppression. This study demonstrated the loss of TQ1 with progression of the disease to Richter's syndrome or to prolymphocytic transformation.[88]

T-CLL makes up less than 5% of all CLL. It is a poorly defined entity in that some of the reported cases appear to represent other chronic lymphoproliferative diseases such as prolymphocytic leukemia (PLL), adult T-cell leukemia/lymphoma, "lymphosarcoma cell leukemia," and chronic Tγ-lymphoproliferative disease.[89–92] Therefore, if this entity is to have diagnostic and clinicopathologic significance, the term "T-cell lymphocytic leukemia" should probably be restricted to those lymphoid proliferations that are morphologically composed of small lymphocytes having scant amounts of agranular cytoplasm and nuclei exhibiting condensed chromatin with slight to moderate nuclear membrane irregularities (clefting and protrusions) and inconspicuous nucleoli. Clinically, T-CLL has a variable presentation, unlike B-CLL, and usually affects older adults.[90,92,93] A helper/inducer phenotype is characteristic ($CD2^+$, $CD3^+$, $CD4^+$), but a cytotoxic/suppressor phenotype has been reported ($CD3^+$, $CD8^+$, $CD4^+$), and these cases have had large amounts of cytoplasm with azurophilic granules, possibly representing Tγ-lymphoproliferative disease.[92,93] Recently, a variant of cytotoxic/suppressor T-CLL was reported that occurs in younger adults and is clinically aggressive with distinct morphologic features.[94]

Chronic Tγ-lymphoproliferative disease

Chronic Tγ-lymphoproliferative disease has also been termed "granulated T-cell lymphocytosis with neutropenia," "chronic T-cell lymphocytosis with neutropenia," "T-suppressor-cell chronic lymphocytic leukemia," and "leukemia of large granular lymphocytes." Although many names have been given to the process, they all describe a distinct clinicopathologic entity characterized by mild-to-moderate peripheral-blood and bone-marrow granular lymphocytosis, neutropenia, splenomegaly, and absence of lymphadenopathy, with an indolent course.[95–99] The T lymphocytes are large granular lymphocytes that typically demonstrate Fc receptors for IgG and are thus termed "Tγ lymphocytes." They have a post-thymic cytotoxic-suppressor T-cell phenotype ($CD2^+$, $CD1^-$, $CD3^+$, $CD8^+$, $CD4^-$) in greater than 95% of the cases, with many expressing the natural killer-cell-associated antigen HNK-1 or Leu-7 (CD57).[97–101] Functionally, these cells display antibody-dependent cell cytotoxicity with variable natural killer activity.[96,99–101] Biologically, this disease appears to be heterogeneous since some patients have clonal chromosomal abnormalities and clonal T-cell receptor gene rearrangements suggesting a neoplastic process, whereas others have experienced spontaneous regression of the disease.[96,102–104]

Prolymphocytic leukemia

The clinical and hematologic features of PLL were first described by Galton et al in 1974.[105] The presenting features typically include a high leukocyte count (usually $>100 \times 10^9$/L with >55% prolymphocytes), massive splenomegaly with variable hepatomegaly, and absent or minimal lymphadenopathy.[106–108] PLL is an aggressive disease and was once considered to be a variant of CLL but is now recognized as a separate entity, based not only on clinical and morphologic characteristics but also on surface-marker studies. Most PLLs are of B-cell lineage and display monoclonal SIg. In contrast to the small lymphocyte of CLL, the prolymphocyte has high-density SIg, which is usually IgM and/or IgD, demonstrates poor spontaneous MRBC rosette formation, and does not express the T1 antigen.[106,107,109,110] As expected, B-PLL will also express some B-cell-restricted surface antigens, such as CD24, CD19, and CD20.

Catovsky et al[111] have found 19% of their PLL cases to be of T-cell lineage. T-PLL is morphologically indistinguishable from B-PLL and initially appeared to have similar biologic behavior.[106,112] However, as additional cases were recognized, it was found that T-PLL was a more aggressive disease having a median survival of seven months, compared to 24 months for B-PLL, and the distinguishing clinical features of lymphadenopathy and cutaneous involvement.[111] It has a mature post-thymic phenotype characterized by the expression of CD5, CD2, CD3, CD4, and/or CD8 antigens and an absence of TdT.[111,113,114] Most cases have a helper-inducer phenotype, and the coexpression of CD4 and CD8 in some cases reflects phenotypic heterogeneity, suggesting that some T-PLL may be derived from early post-thymic T lymphocytes.

Prolymphocytic transformation of CLL represents

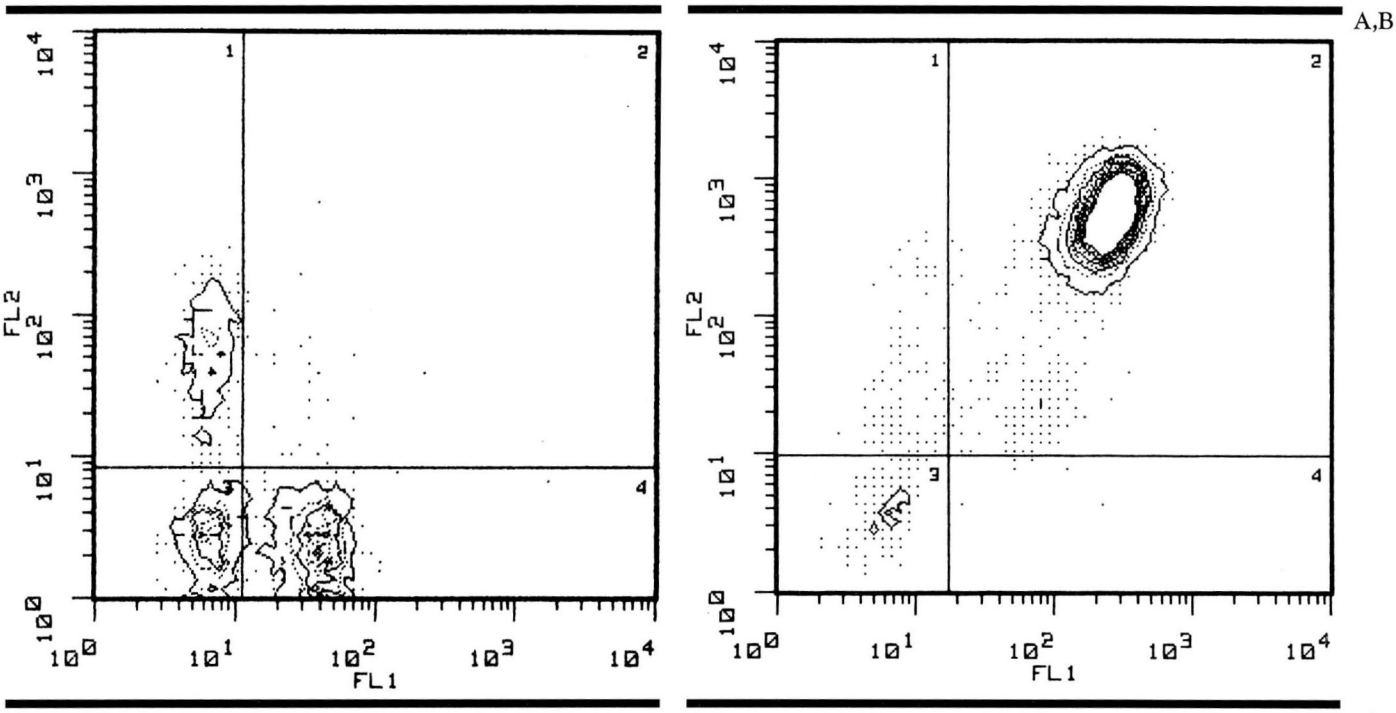

FIGURE 7. Two color flow-cytometric histograms of HCL from peripheral blood. FIGURE 7A. The hairy cells are CD19$^+$ (FL1) and CD5$^-$ (FL2), in contrast to CLL in which these antigens are coexpressed. Note the normal CD5$^+$ T cells in quadrant 1. FIGURE 7B. The coexpression of CD22 (FL1) and CD11c (FL2) is a characteristic feature of HCL.

progression to a more aggressive form of the disease and is defined by a progressive increase in circulating prolymphocytes (>15%), enlarging spleen and/or regional lymphadenopathy, anemia, and thrombocytopenia. Interestingly, this emerging population of prolymphocytes has immunologic characteristics of both CLL and PLL in that it has intermediate- to high-density SIg, a high percentage of MRBC rosettes, and variable expression of CD5.[107,115,116] It has been postulated that this transformation represents a clonal evolution of CLL, and this is supported by the preservation of the same SIg isotype and light-chain type in the prolymphocyte population as that of the chronic-phase CLL.[116] The concept of clonal evolution has also been advanced by other reports in which there has been cytogenetic or immunologic evidence of clonal transformation from T- or B-CLL to large-cell lymphoma.[117–119]

Hairy-cell leukemia

Leukemic reticuloendotheliosis or hairy-cell leukemia (HCL) was originally described by Bouroncle et al in 1958[120] as a neoplasm of the reticuloendothelial system. It has been well characterized clinically as a disease afflicting middle-aged men who typically present with fatigue, splenomegaly, and pancytopenia. The bone marrow, peripheral blood, and spleen are involved by leukemic cells that demonstrate the almost pathognomonic morphologic feature of serrated cytoplasmic membranes with delicate hair-like projections.[121] A subgroup (type II variant) has also been identified that has high white-cell counts and unique ultrastructural and phenotypic features intermediate between HCL and PLL.[122,123]

For many years, the cell of origin for HCL was unknown; numerous conflicting reports in the literature suggested that it was of monocyte/histiocyte, lymphoid, or even myeloid origin. However, it has recently been shown with gene-rearrangement studies that HCL is unquestionably a B-cell lymphoid neoplasm.[124] Surface-marker profiles also support the B-cell lineage of this process. Those B-cell-associated antigens consistently identified on hairy cells include HLA-DR, CD19, and CD20 (*Figure 7A*).[124–127] CD10 and CD21 antigens are absent, but SIg and PCA-1 are present in many cases. Jansen et al[128] have noted prolonged survival in those cases expressing kappa light chains. PCA-1 is associated with the terminal secretory stage of B-lymphocyte development (plasma cells), suggesting that HCL is

A,B

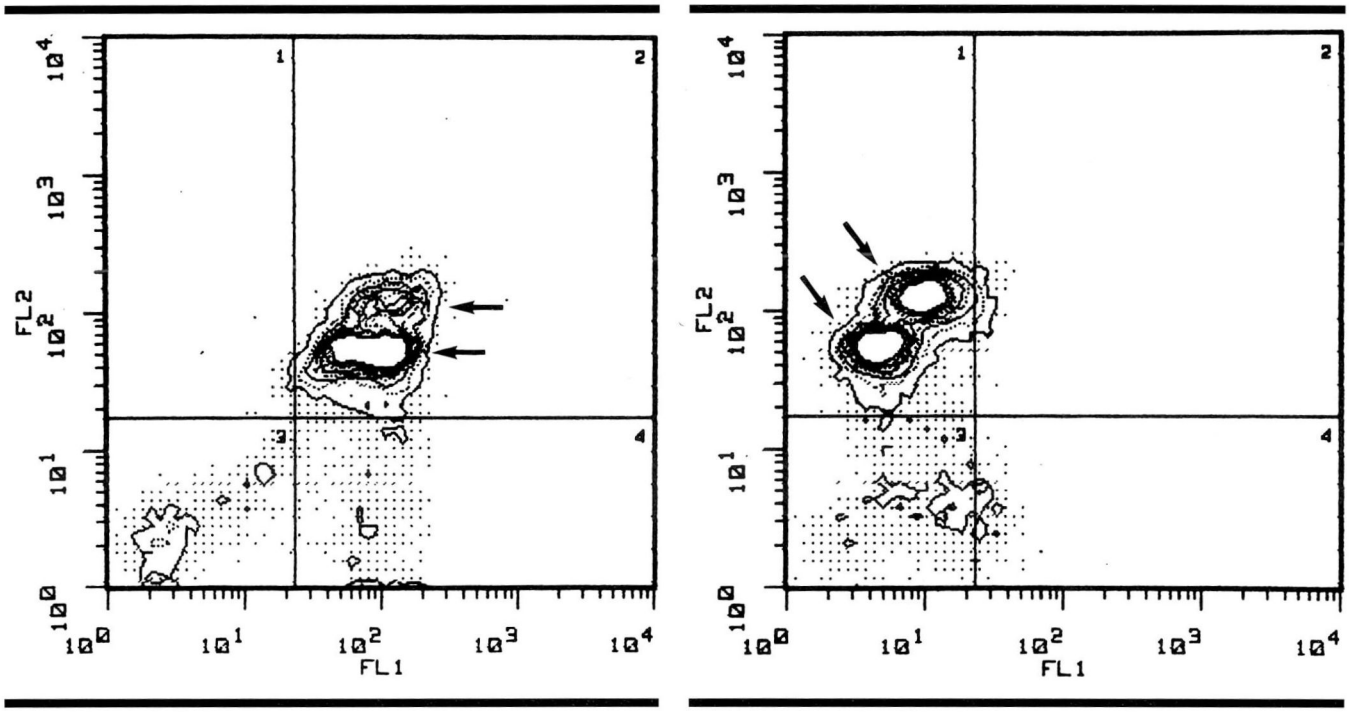

FIGURE 8. Two color flow-cytometric histograms of Sezary's syndrome from peripheral blood. Note the two populations of $CD4^+$ cells in both histograms (arrows), indicating different levels of fluorescence intensity or antigen expression. FIGURE 8A. The Sezary cells coexpress CD3 (FL1) and CD4 (FL2). FIGURE 8B. Deletion of CD7 (FL1) with CD4 (FL2) expression.

derived from presecretory B cells with a maturation arrest occurring between the mature B-cell and plasma-cell stage.[126] In contrast, CD38, another plasma-cell-associated antigen, is absent.[127] CD25 (IL-2 receptor) is also expressed by most HCLs and is acquired by splenic B lymphocytes after stimulation.[124] Therefore, it has been suggested that hairy cells expressing this antigen may represent activated neoplastic B cells. Myeloid markers with which hairy cells will also react inconsistently include CD11b and CDw13 (My7).[124–126]

Recently, two monoclonal antibodies against HCL cells were raised, CD11c (Leu-M5) and CD22 (Leu-14). The first has monocyte/macrophage specificity but also reacts with most HCL, while the second is a pan-B-cell marker present in high concentration on hairy cells.[129,130] The identification of both CD11c and CD22 on a neoplastic lymphoid-cell population having the characteristic morphologic features is consistent with HCL, but other lymphoproliferative disorders, such as CLL and circulating lymphomas, may coexpress these antigens (*Figure 7B*).[131] I recommend using two-color flow-cytometric analysis when this diagnosis is in the differential. Single-color analysis may be performed, but caution must be exercised in the interpretation of data if a significant number of monocytes contaminate the cell suspension.

Leukemic phase of non-Hodgkin's lymphoma

The leukemic phase of non-Hodgkin's lymphoma (LP-NHL), inappropriately termed "lymphosarcoma-cell leukemia," represents involvement of the peripheral blood by lymphoma cells.[132–134] This may occur at any time during the course of NHL and may be detected with sensitive immunologic techniques in more than 30% of the cases without a leukemic phase. Therefore, LP-NHL must be considered in the differential diagnosis when any atypical lymphoid-cell population is found in the blood. In general, peripheral-blood involvement is directly related to the extent of marrow infiltration by the neoplastic cells and is more frequently associated with certain morphologic subtypes, such as follicular small-cleaved cell (50%), small lymphocytic (56%), and lymphoblastic lymphomas (50%).[135–141] Large-cell lymphomas infrequently infiltrate the bone marrow, but when present, usually also involve the blood and are often associated with unresponsive disease.[137] Histologic subtype has been shown to be the most important parameter in assessing prognosis in LP-NHL.[141]

TABLE 6
IMMUNOPHENOTYPIC CHARACTERISTICS OF THE B-CELL CHRONIC LYMPHOID LEUKEMIAS

	Differentiation antigens				
	CD5	SIg	CD19	CD22*	CD11c*
CLL	+	+	+	+	–
PLL	–	++	+	+	–
HCL	–	+	+	++	++
LP-NHL†	±	++	+	+	–

*CLL and some NHL may coexpress CD22 and CD11c.
†Small lymphocytic and intermediate cell type non-Hodgkins lymphomas may express CD5.
CLL = chronic lymphocytic leukemia, *PLL* = prolymphocytic leukemia, *HCL* = hairy-cell leukemia, and *LP-NHL* = leukemic phase of non-Hodgkin's lymphoma.
++ = high-density antigen.

TABLE 7
COMMON IMMUNOPHENOTYPES OF THE T-CELL CHRONIC LYMPHOID LEUKEMIAS

	Common immunophenotype
ATL	$CD2^+$ $CD5^+$ $CD3^+$ $CD4^+$
CLL	$CD2^+$ $CD5^+$ $CD3^+$ $CD4^+$
Tγ-LPD	$CD2^+$ $CD3^+$ $CD8^+$ $CD4^-$ $CD57^+$
PLL	$CD2^+$ $CD5^+$ $CD3^+$ $CD4^+$
LP-NHL*	$CD2^+$ $CD5^+$ $CD3^+$ $CD4^+$
Sezary cells†	$CD2^+$ $CD5^+$ $CD3^+$ $CD4^+$ $CD7^-$ $Leu8^-$

*Lymphoblastic lymphomas are not included. Antigen deletions are common—CD5 and CD7 > CD3 > CD2.
†CD7 and Leu-8 are frequently deleted in mycosis fungoides (Sezary's syndrome), while CD2 and CD1 are less commonly absent.
ATL = adult T-cell leukemia-lymphoma, *CLL* = chronic lymphocytic leukemia, *Tγ-LPD* = chronic Tγ-lymphoproliferative disease, *PLL* = prolymphocytic leukemia, and *LP-NHL* = Leukemic phase of non-Hodgkin's lymphoma.

Most LP-NHLs are B-cell neoplasms, and flow cytometric analysis can be of value in detecting circulating monoclonal B lymphocytes.[135,136,140–142] Peripheral T-cell lymphomas are more difficult to identify in the blood, especially at low levels, but may be accomplished provided antigen deletions are identified. Immunocytochemical evaluation of cytospin preparations may be more convenient and efficacious in identifying LP-NHL cells, depending upon their concentration, since staining of the atypical cells can be directly visualized. Immunophenotypic concordance between the neoplastic lymphocytes in the blood and lymph-node biopsy further support a diagnosis of LP-NHL.

Mycosis fungoides is a cutaneous T-cell lymphoma deserving special mention. Circulating lymphoma cells (Sezary cells) are found in approximately 25% of the cases (Sezary syndrome)[143] and have distinctive morphologic and immunophenotypic characteristics. The cells are intermediate in size and have high nuclear-cytoplasmic ratios with only a thin rim of blue cytoplasm. The nuclei are convoluted and have coarse dark chromatin and inconspicuous nucleoli. Surface-marker studies reveal these cells to have a helper phenotype ($CD3^+$, $CD4^+$) with frequent deletions of CD7 and Leu8 antigens while the pan-T cell markers CD5 and CD2 are only occasionally deleted (*Figure* 8).[144,145]

The immunophenotypic characteristics of the B- and T-cell chronic lymphoid leukemias are summarized in *Tables* 6 and 7.

SUMMARY

The ability to recognize, diagnose, and subclassify lymphocytic leukemias has been significantly enhanced by the development of monoclonal antibodies and immunologic techniques that can be applied in the clinical laboratory. The more precise subclassification of lymphoid leukemias based on their immunotype and their relationship to normal T- and B-lymphocyte development has increased understanding of their ontogeny and the ability to identify clinically significant subtypes not previously recognized. Immunophenotyping should be considered an adjunct to routine morphological and cytochemical evaluation. This cannot be overemphasized since it is critical that any case being considered for immunotyping have a tentative morphologic diagnosis so that a panel of immunologic markers can be intelligently selected for analysis. Immunotyping should not be used as the primary means of making a diagnosis, nor should it be applied for diagnostic purposes in a clinical laboratory before gaining considerable experience with the methodology and with the immunologic markers to be used.

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