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Immunophenotypic analysis of acute lymphocytic leukemia

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The identification and quantitation of cellular antigens through fluorochrome-labeled monoclonal antibodies ("immunophenotyping") is one of the most important applications of the flow cytometer. The immunophenotypic identification and classification of cells by flow cytometry began in the early 1980s with the revolution in immunology that brought about the discovery of T- and B-cells. This technology has now expanded to the analysis of other cells such as monocytes, macrophages, myeloid stem cells, tumor cells and other cell types. Clinically, immunophenotypic analysis is a critical part of the initial diagnosis and classification of the acute leukemias since the primary basis of treatment strategy depends upon antigenic parameters. In addition, immunophenotypic analysis of the acute leukemias provides prognostic information not available by other techniques, provides a sensitive means to monitor the progress of patients after chemotherapy or bone marrow transplantation, and aids in detection of minimal residual disease [1]. In addition to immunophenotype, a number of other flow cytometric parameters are under evaluation that may provide additional diagnostic or prognostic information. These include measurements of apoptosis, multidrug resistance, leukemia-specific chimeric proteins, cytokine receptors, etc. The major technical and clinical issues in the immunophenotypic analysis of acute lymphocytic leukemia are addressed in this chapter. Other reviews of this subject have been recently published [2–9].

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Immunophenotypic analysis of acute leukemia

Leukemias represent abnormal proliferations of hematopoietic cells that are arrested at a discrete stage of differentiation. Leukemias are classified into acute and chronic forms based on a constellation of clinical and laboratory findings. The acute leukemias are classifed into two subclasses: the lymphoblastic (ALL) type and non-lymphoblastic (ANLL) type, based on morphologic, cytochemical and immunophenotypic features. Each type is further subdivided into prognostically and therapeutically relevant subtypes based on additional immunologic, cytogenetic and molecular features. Unfortunately, the ANLLs have proven to be heterogeneous and more difficult than the ALLs to classify into precise subgroups.

Acute lymphoblastic leukemia (ALL) is the most common cancer in children. It also is seen in adults, where it accounts for approximately 20% of acute leukemias. A high rate of success recently has been achieved in treating childhood cases of ALL, but adult cases are generally more resistant to therapy.

Leukemic cells do not show abnormal morphologic changes in the same manner as other tumors; therefore, the definitive diagnosis of leukemia or other hematopoietic neoplasm can be made only by observing a shift in the distribution or maturity of a cell population. Nearly three decades ago, the French-American-British (FAB) group recognized the presence of increased immature hematopoietic precursors in the acute leukemias. This resulted in a morphologic and cytochemical criteria for subdividing ALL into three subtypes, and acute myelogenous leukemia (AML) into seven subtypes.

The subsequent development of the flow cytometer provided medical investigators with a powerful tool that rapidly led to advances in knowledge of the biology of the acute leukemias. The flow cytometer was an ideal tool to apply monoclonal antibodies to the study of human disease. To date, 247 classes (cluster designations [CD]) of monoclonal antibodies have been discovered which recognize distinct human differentiation antigens. Many of these monoclonal antibodies, labeled with various fluorochromes, are commercially available in large quantities. The flow cytometer also permits the study of large numbers (typically 10,000 to 30,000) of individual cells in a short period so that objective, quantitative data is obtained. Furthermore, many different cellular parameters can be simultaneously detected and recorded (ie, multiparametric analysis), and different cell populations can be separated or sorted in a functionally intact state for further study. Very small numbers of leukemic cells also can be detected, far beyond the sensitivity of conventional techniques. The analysis of small numbers of cells is important, not only in the initial diagnosis of leukemia, but also in treated patients, where the presence of residual or recurrent leukemia can be detected before the reappearance of clinical symptoms. The recent World Health Organization (WHO) classification of the acute leukemias, published in the monograph *Pathology and Genetics of Tumors of the Haematopoietic and Lymphoid Tissues*, incorporates nonmorphologic data, including flow cytometry, and is followed in this article (Fig. 1) [10].

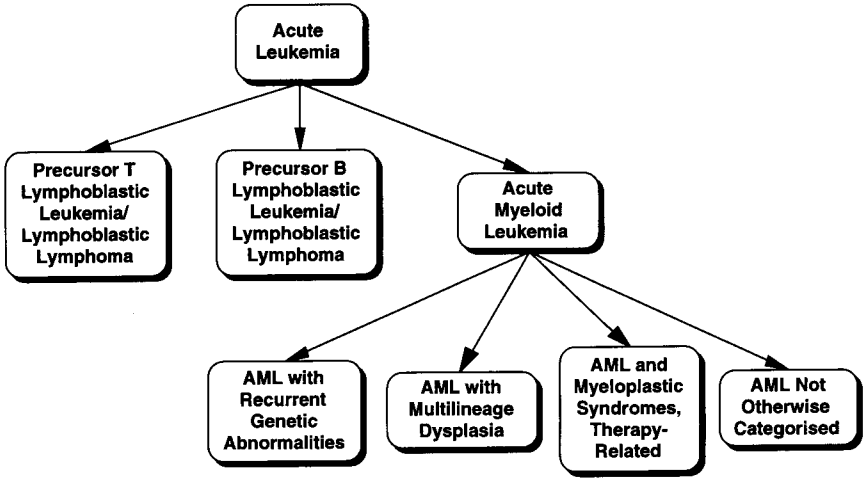

Fig. 1. World Health Organization classification of acute leukemia, 2001.

Flow cytometric analysis of large numbers of patients with hematopoietic neoplasms revealed either maturation shifts or distribution shifts in blood cells. A "maturation shift" is the presence of a discrete population of cells expressing immature surface antigens that normally disappear when the cell reaches the mature state. Such a finding in hematopoietic cells suggests that the cells have arrested at one stage of development. One such differentiation antigen is CD10 (cALLA), which is present on B-lymphocytes very early in development, but is later lost. Since CD10+ cells are present in fewer than 16/1,000,000 cells in the peripheral blood of normal individuals, CD10 has proven to be a very useful marker for the diagnosis of precursor B-cell acute lymphoblastic leukemia (ALL) and for detecting early relapse in precursor B-cell ALL. Another example is CD1a that is present on thymocytes only during the phase when the cells exhibit intermediate differentiation. The presence of large numbers of these cells in the peripheral blood is very useful in the diagnosis of precursor T-cell ALL. A maturation shift also can be detected by the presence of an early surface marker that normally persists on the mature cells, while later surface markers are absent. An example of this type of maturation shift is seen in B-lymphocytic malignancies that demonstrate CD19 but lack CD20.

A "distribution shift" is the presence of a marked expansion of a population of cells expressing mature surface antigens. Thus, the presence of a large population of B lymphocytes with IgGκ surface markers is indicative of malignancy if cells with IgGλ surface markers are present in normal numbers or suppressed. This concept of "monoclonality" forms the basis for the diagnosis of many hematopoietic neoplasms. Distribution shifts are very helpful when the cell population in question is normally low in number, (eg, B-lymphocytes in the peripheral blood [about 20% of the lymphocytes] or in the lymph node [about 40% of the lymphocytes]). Thus, if 95% of the peripheral

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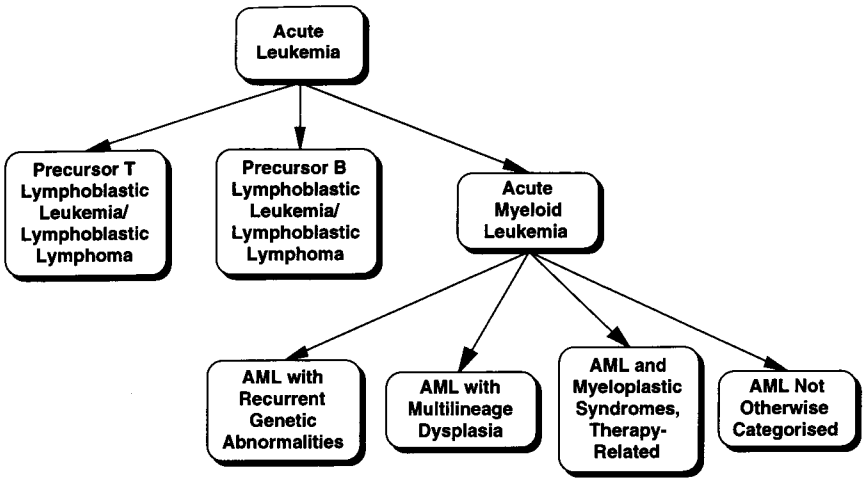

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blood lymphocytes are immunophenotypically identified to be B-lymphocytes, one can assume a diagnosis of malignant lymphoproliferative disorder of B-cell type until proven otherwise. The interpretation of distribution shifts is much more difficult if the cell population in question normally comprises the great majority of the cells; eg, T-lymphocytes in the peripheral blood (about 80% of the lymphocytes). Thus, if 95% of the peripheral blood lymphocytes are immunophenotypically identified to be T-lymphocytes, one cannot assume a diagnosis of malignant lymphoproliferative disorder of a T-cell type unless other data are in support of such a diagnosis. The increase in the percentage of the T cells could simply be within the reference range or represent a response to an inflammation. Distribution shifts are much more suggestive of malignancies when the increase in percentage of a cell population is also associated with a marked increase in the absolute number of those cells, eg, 95% of the peripheral blood lymphocytes exhibit a T-cell phenotype in a patient with a lymphocyte count of 30,000/μL.

Technical considerations in clinical immunophenotypic analysis

The correct immunological classification of an acute leukemia or other hematologic neoplasm requires strict application of the principles of flow cytometric diagnosis as discussed by Dr. McCoy in this issue. Knowledge of the technical capabilities and limitations of the flow cytometer is essential, as well as proper specimen collection and preparation, accurate instrumental analysis, appropriate quality control, accurate data interpretation, and prompt reporting of the results in a format that is complete and easy to comprehend. The requesting physician must understand the clinical utilization of flow cytometry and how the results of an analysis can benefit a particular patient. The laboratory performing the analysis must have well-maintained instrumentation, a thorough and up-to-date procedure manual, an adequate number of well-trained and experienced personnel, quality reagents, and a comprehensive scheme of quality control [11]. Cost-efficient flow cytometric analysis of neoplastic hematologic specimens requires that each request be examined in light of the clinical and laboratory findings and the morphological features of the peripheral blood, bone marrow or other patient specimen. Flow cytometric analysis must then be conducted with consideration of one or more definitive questions to be answered. Finally, the data should be reviewed in light of the other clinical and laboratory findings and additional studies performed or recommended if the initial questions are not answered.

Flow cytometric analysis of any specimen requires the following steps:

- Specimen collection and transportation
- Cell preparation and purification
- Fluorescent probe application (staining)

- Instrument analysis (data collection) and data storage
- Data interpretation and reporting

Specimen collection and transportation

The successful outcome of a flow cytometric study begins with proper specimen collection and prompt delivery of the specimen to the laboratory. To order the appropriate assay and oversee the delivery of the proper specimen, the physician must have complete information regarding the clinical utilization of flow cytometry, specimen requirements, specimen delivery, and the operation of the laboratory performing the analysis. The test requisition, whether written or computerized, should include complete specimen demographic information, the identity of the requesting physician, the identity of the assay being requested, and some basic clinical information. A personal consultation with a pathologist knowledgeable in flow cytometric analysis may be required in complex clinical cases. The person collecting the specimen must be adequately trained and knowledgeable about specimen requirements and any special collection instructions for the assay ordered. Venipuncture should be performed as described in NCCLS standard (H3-A4; Collection of Diagnostic Blood Specimens by Venipuncture), with utmost regard for the safety of the phlebotomist [11,12].

Each specimen container must be labeled with the name and identification number of the patient, the date and time collected, the type of specimen, and the initials of the person collecting the specimen. A completed requisition form or electronic request with pertinent information must accompany the specimen. Since cell autolysis and loss of surface markers occurs after specimen collection, either prompt specimen analysis or preservation for later analysis is required. Generally, cell viability and antigen expression are maintained for at least 24 hours in peripheral blood or bone marrow cells at room temperature in a collection tube containing heparin, ethylenediaminetetraacetic acid (EDTA), or acid citrate dextrose (ACD). Solid tissue and body fluid specimens can also be transported or stored for a short period of time, provided they are diluted with an equal volume of sterile RPMI containing 5% fetal calf serum (RPMI/5% FCS), 100 U/mL of penicillin, and 100 μg/mL of streptomycin [13]. If there is a delay in analysis longer than 24 hours, physical separation of the cell population under investigation and cryopreservation in liquid nitrogen is recommended. Specimen requirements for flow cytometric analysis depend upon the cellularity of the specimen, the viability of the cells in the specimen, and the type of analysis performed. Approximately 1×10^6 cells/μL (100,000 total) are required for each monoclonal antibody (or antibody mixture) utilized in the analysis or for the determination of DNA content. Under conditions of normal cellularity, 10 to 20 μL of peripheral blood, 3 to 4 μL of bone marrow, or 0.5 gm (0.5 × 1 cm) of tissue will provide adequate cells for most immunophenotypic panels. However, since cytogenetic analysis, molecular studies, microbiologic cultures, and other

studies are usually performed in conjunction with flow cytometric analysis, planning is required to assure an adequate total specimen volume and proper sample collection.

Cell preparation and purification

The preparation of a specimen for flow cytometric analysis requires the formation of a single cell suspension, the removal of cells that may interfere with the analysis and the assessment of specimen adequacy. During this process, cell viability and antigenicity must be maintained and the selective loss of cell subpopulations must be avoided. Methods of specimen preparation that minimize manipulation of the specimen are recommended to achieve these goals.

Fluorescent probe application

All immunofluorescent staining procedures require knowledge of several factors that may influence the results. Assuming proper specimen collection, storage and preparation for staining, the cells are stained with properly chosen fluorescent-labeled monoclonal antibodies and incubated under appropriate conditions. Dual-color immunophenotypic analysis is employed by some laboratories for flow cytometric analysis of immunophenotypic specimens, but three- or four-color analysis is highly recommended for cost efficiency and accurate analysis of complex cell populations [2].

Generally, the laboratory should use the minimal number of monoclonal antibodies to answer the clinical question under consideration. Unfortunately, each patient is unique and there is no single staining panel for every clinical circumstance. Monoclonal antibody selection for hematologic neoplasms requires review of clinical information and other laboratory data, the specimen quantity and quality, available laboratory resources, and other factors. For example, monoclonal antibodies specific for CD3, CD7, CD10, CD13, CD14, CD19, CD33, CD34, CD45 and HLA-DR antigens are adequate for the identification of most acute leukemias [14,15]. However, additional antibodies are sometimes necessary. Antibodies specific for T-cell antigens (ie, CD1a, CD2, CD5, CD4, CD8) should be included in the presence of a mediastinal mass or suggestive cellular features such as nuclear cleaving. Platelet-specific antibodies (ie, CD41, CD61) should be used in the case of a leukemia with undifferentiated or "megakaryoblastic" features. Antibodies specific for surface heavy and light chains should be included for a case with FAB-L3 morphology. Other B-cell-specific antibodies (ie, anti-CD20, -CD22, -CD79a, and -IgM) may be necessary to evaluate some B-ALLs. Other helpful antibodies include those that recognize CD16, CD56, CDw65, CD117, TdT and cytoplasmic CD3. Anti-glycophorin-A or anti-CD36 antibodies are extremely helpful in bone marrow specimens containing erythroblasts, which are difficult to separate physically or electronically from immature leukocytes. The simultaneous analysis of cell-surface and intracellular antigens can be performed if the cell membrane is permeabilized before the addition of flourochrome-labeled monoclonal antibodies [16–31]. Unfortunately, the routine analysis of intracellular antigens by clinical flow cytometry laboratories has been limited by technical difficulties, poor reproducibility between laboratories, and difficulty in interpretation of the results. Table 1 is a summary of the antigens commonly studied in flow cytometric immunophenotypic analysis of hematopoietic neoplasms.

Instrument analysis and data storage

The analysis of a fluorochrome-labeled single cell suspension requires the following steps:

- Turn on laser, computer, and electronics.
- Check system pressure and vacuum gauges.
- Check optical alignment, fluorescence standardization and color compensation of instrument.
- Test antibody integrity by verifying quality control samples.
- Prepare and run specimens.
- Analyze and store all data.

In the flow cytometer, labeled cells or other particles (microorganisms, chromosomes, coated beads, etc) are aspirated by a sample probe into a flow chamber where, under slight pressure, they are directed into a stream of fast moving sheath fluid (non-fluorescent saline solution). The pressure of the sheath fluid against the cell suspension aligns the cells in single file (hydrodynamic focusing). Inside the flow chamber, the cells pass through a sensing area where a laser beam (argon ion, krypton, helium-cadmium, helium-neon, etc) or other high intensity light source (mercury arc, etc) is precisely focused. The intensity of light deflected from the cell surface (forward angle light scatter [FALS]) and internal structures or granules (side scatter [SS] or right angle light scatter [RALS]) gives information about cell size, morphology, internal structure, viability and granularity. Any fluorescent dyes attached to the cells are excited by the laser light energy and emit fluorescence. Several photodiodes placed at different angles to the detection zone measure light intensity and the resulting electrical signals are amplified and passed to a computer for further processing (Fig. 2). This process is accomplished at very low flow rates (typically 5000 cells/second).

Graphical representation of flow cytometric data is necessary to determine the number and interrelationship of the cell populations which are present, to assess the adequacy of the information, and to select the appropriate method for additional graphical or statistical analysis. The basic forms of graphic representation in flow cytometry include the two dimensional display (X-Y display, scatter gram, "dot plot") and histogram (frequency distributions) (Fig. 3).

Multiparametric analysis is one of the most important capabilities of the flow cytometer. At present, nearly all flow cytometers in clinical laboratories have the

Table 1
Characteristics of human leukocyte antigens for hematologic diagnosis

Antigen	Normal cellular expression	Major diagnostic application	Biological function	Representative commercial antibodies
CD1a	Cortical thymocytes, dendritic reticular cells, langerhans cells	Precursor T-cell ALL and some lymphoblastic lymphomas.	MHC class I-like molecule, associated with β-2-microglobulin. May have specialized role in antigen presentation, including delivery of signals for lymphocyte activation. Postulated role in thymic T-cell development.	T6, Leu6
CD2	T cells, thymocytes, NK cells	Hematopoietic neoplasms of T-cell lineage.	Sheep erythrocyte rosette receptor. LFA-3 (CD58) ligand. Adhesion molecule, can activate T-cells.	T11, Leu5b, 9.6
CD3	T cells, thymocytes	Hematopoietic neoplasms of T-cell lineage.	Associated with the T-cell antigen receptor. Required for cell surface expression of and signal transduction by TCR.	T3, Leu4
CD4	Thymocyte subsets, helper and inflammatory T cells, monocytes, macrophages	Sezary cell leukemia and some hematopoietic neoplasms of T-cell lineage.	Coreceptor for MHC class II molecules. Binds lock on cytoplasmic face of membrane. Receptor for HIV-I and HIV-2 gp120.	T4, Leu-3a
CD5	T cells, thymocytes, B cell subset	B-CLL and most hematopoietic neoplasms of T-cell lineage.	CD72 ligand Signal transduction through antigen-specific receptor complex. T-cell activation,	T1, Leu1, T101, 10.2
CD7	Pluripotential hematopoietic cells, thymocytes, major T-cell subset, NK cells, early myeloid cells,	Hematopoietic neoplasms of T-cell lineage.	T-and NK-cell activation.	Leu9, 3A1, WT 1
CD8	Cytotoxic T cells, thymocyte subsets, NK cells	Hematopoietic neoplasms of T-cell lineage.	Coreceptor for MHC class I molecules. Binds lck on cytoplasmic face of membrane. Regulates function of CD3/TCR complex	T8, Leu2a
CD10	Early B lymphocytes, PMNs B- and T-cell precursors, bone marrow stromal cells	Precursor B-cell ALL and non-Hodgkin lymphomas of follicular cell center origin.	CALLA. Zinc metalloproteinase. Neutral endopeptidase	CALLA, J5
CD11b	Monocytes, granulocytes, NK cells	Myelomonocytic leukemias, particularly of FAB M4 and M5 subclasses.	Mac-1. Cell adhesion molecule. Subunit of integrin CR3 (associated with CD18). Binds CD54, complement component iC3b and extracellular matrix proteins.	MAC-1, CR1, Mo1, OKM1
CD11c	Myeloid cells, monocytes	Hairy cell leukemia and related hematopoietic neoplasms.	Cell adhesion molecule. Subunit of integrin CR3 (associated with CD18). Binds fibrinogen	Leu-M5
CD13	Myelomonocytic cells	Leukemias of myeloid lineage.	Zinc metalloproteinase. Aminopeptidase N	My7, LeuM7
CD14	Myelomonocytic cells	Myelomonocytic leukemias, particularly of FAB M4 and M5 subclasses.	Receptor for complex of LPS and LPS binding protein (LBP)	LeuM3, Mo2, My4
CD15	Granulocytes, monocytes, endothelial cells	Hodgkin's lymphoma, other hematopoietic neoplasms.	Lewis-x (Lex) antigen. Branched pentasaccharide, expressed on glycolipids and many cell surface glycoproteins. Sialylated form is a ligand for CD62E (ELAM).	Leu M1, My1
CD16	NK cells, granulocytes, macrophages	Hematopoietic neoplasms of NK-cell lineage.	FcgRIII. component of low affinity Fc receptor (FcgRIII). Mediates phagocytosis and ADCC.	OKNK, Leu-11a,b,c
CD19	Pan B-cell antigen	Precursor B-cell ALL and non-Hodgkin lymphoma of B-cell lineage.	Forms complex with CD21 (CR2) and CD81 (TAPA-1). Coreceptor for B-cells. Regulation of B-cell activation	B4, Leu12
CD20	B-cell antigen	Precursor B-cell ALL and non-Hodgkin lymphoma of B-cell lineage.	Ca++ channel, B-cell activation?	B1, Leu16
CD21	Mature B cells, follicular dendritic cell	Leukemia and lymphoma diagnosis.	C3d/EBV-receptor (CR2). Coreceptor for B-cells (with CD19 and CD81)	B2, CR2

(continued on next page)

Table 1 (*continued*)

Antigen	Normal cellular expression	Major diagnostic application	Biological function	Representative commercial antibodies
CD22	Mature B cells	Leukemia and lymphoma diagnosis. Cytoplasmic CD22 is B cell lineage-specific antigen.	Cell adhesion molecule. Ig-mediated adhesion of B-cells monocytes, T cells.	B3, Leu14, SHC11
CD23	Activated B cells, activated macrophages, eosinophils, follicular dendritic cells, platelets	Leukemia and lymphoma diagnosis.	Low affinity receptor for IgE (Fc^{+}RII). Ligand for CD19:CD21:CD81 coreceptor.	Leu-20, B6
CD24	B cells, granulocytes	Leukemia and lymphoma diagnosis.	Possible human homologue of mouse Heat Stable Antigen (HSA) or J11d.	BA-1
CD25	Activated T cells, activated B cells, monocytes	Hairy cell leukemia, ATL/L, other hematopoietic neoplasms.	TAC Interleukin 2 receptor alpha chain.	TAC
CD30	Activated B-and T-cells	Hodgkin's lymphoma, anaplastic large cell lymphoma.	Ki-1 Growth factor receptor.	Ki-1, Ber-H2
CD33	Myeloid cells myeloid progenitor cells, monocytes	Leukemias of myeloid lineage.	Sialic acid adhesion molecule.	My9, LeuM9, L4F3
CD34	Hematopoietic precursors, capillary endothelium	Leukemias of early myeloid lineage, lymphoblastic lymphoma.	Ligand for CD62 (L-selectin).	HPCA-2, My10
CD36	Platelets, mature monocytes and macrophages, microvascular endothelial cells, mammary endothelial cells, during stages of erythroid cell development and on some macrophage derived dendritic cells.	Erythroleukemia (AML, FAB-M6). Reticulocyte pathophysiology.	Glycoprotein IV. Cell adhesion molecule in platelet adhesion and aggregation, platelet-monocyte and platelet-tumor cell interaction. Scavenger receptor for oxidized LDL and shed photoreceptor outer segments. Recognition and phagocytosis of apoptotic cells. Cytoadherence of Plasmodium falciparum-infected erythrocytes.	FA6-152
CD38	Early B- and T-cells, activated T cells, germinal center B cells, plasma cells	Plasma cell dyscrasias, some non-Hodgkin's lymphomas.	Leukocyte activation.	Leu17, T10
CD41	Megakaryocytes, platelets	Acute leukemia of megakaryocytic lineage (AML, FAB-M7).	aIIb integrin. Associates with CD61 to form GPIIb. Binds fibrinogen, fibronectin, von Willebrand factor and thrombospondin	GpIIbIIIa, PL-273
CD42b	Megakaryocytes, platelets	Acute leukemia of megakaryocytic origin (AML, FAB-M7).	GPIb, vWF receptor. Binds von Willebrand factor and thrombin. Essential for platelet adhesion.	GP1b, FMC-25
CD43	T cells, myeloid cells, some B cell lymphomas	Some T-cell lymphoproliferative diseases.	Leukosialin, sialophorin binds CD54 (ICAM-1).	Leu22
CD45	Panhematopoietic	All hematopoietic neoplasms.	Leukocyte common antigen. Tyrosine phosphatase, augments signalling through antigen receptor of B-and T-cells.	T200, LCA
CD45RA	B cells, T-cell subsets (naive T-cells) monocytes		Isoforms of CD45 containing the A exon.	2H4
CD45RO	T-cell subsets, B-cell subsets, monocytes, macrophages		isoform of CD45 containing none of the A, B and C exons.	UCHL-1
CD55	Widespread cellular distribution, hematopoietic and non-hematopoietic cells	Absent or deficient in paroxysmal nocturnal hemoglobinuria (PNH).	Decay Accelerating Factor (DAF). Complement activation inhibitor. Binds C3b, disassembles C3/C5 convertase	DAF
CD56	NK cells	Hematopoietic neoplasms of NK-cell lineage.	NKH-1. Cell adhesion molecule. Isoform of Neural Cell Adhesion Molecule (NCAM).	NKH-1, Leu19, N-CAM
CD57	NK cells, subsets of T cells, B cells and monocytes	Hematopoietic neoplasms of NK-cell and T-cell lineage.	HNK-1. Oligosaccharide, found on many cell surface glycoproteins.	HNK-1, Leu7
CD59	Many hematopoietic cells	Absent or deficient in paroxysmal nocturnal hemoglobinuria (PNH).	Protectin, Mac inhibitor. Complement inhibitor. Binds comlement components C8 and C9 to block assembly of membrane attack complex.	MIRL
CD61	Megakaryocyte platelets, megakaryocytes, macrophages	Acute leukemia of megakaryocytic origin (AML, FAB-M7).	Integrin b3 subunit, associates with CD41 (GPIIb/IIIa)(fibrinogen receptor) or CD51 (vitronectin receptor).	Plt GPIIIa, 10-P61, Y2/51

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Table 1 (*continued*)

Antigen	Normal cellular expression	Major diagnostic application	Biological function	Representative commercial antibodies
CD79a	B cells (lineage specific)	Hematopoietic neoplasms of B-cell lineage.	Components of B cell antigen receptor, required for cell surface expression and signal transduction.	Mb-1
CD95	Widespread distribution		Apo-1, Fas, TNF-like ligand. Transmits apoptosis signal.	APO-1, Fas
CD103	Intestinal epithelial lymphocytes Intraepithelial lymphocytes, 2–6% of peripheral blood lymphocytes		aE integrin.	HML-1
CD117	Blast cells of myeloid lineage, mast cells	Acute myeloid leukemias.	c-kit. Stem Cell Factor (SCF) receptor. Critical for stem cell survival and progenitor cell replication/differentiation	17F11, 95C3, YB5.BB
HLA-DR	B cells, monocytes, activated T cells, myeloid precursors	Hematopoietic neoplasms	HLA Class II receptor.	HLA-DR. Ia
Glycophorin A	Erythrocytes, erythroid precursors	Erythroleukemia (AML, FAB-M6).		Glycophorin A, 10F7
TdT	Lymphoblasts, thymocytes, myeloblast subset	Acute leukemia and lymphoblastic lymphoma		TdT
Myeloperoxidase				

Data from Protein Reviews on the Web. CD MOLECULES. Human cell surface molecules recognized by the International Workshops on Human Leukocyte Differentiation Antigens.http://www.ncbi.nlm.nih.gov/PROW/guide/45277084.htm.

Fig. 2. Immunophenotypic analysis of an acute leukemia by flow cytometry. (a) Fluorochrome-labeled monoclonal antibody solutions are added to a cell suspension from a peripheral blood, bone marrow aspirate, or lymph node. The tubes are incubated at room temperature for a short period. (b) The labeled cell suspensions are passed through the flow cell of a flow cytometer. Many flow cytometers are automated, but some models require the operator to process the tubes individually. More than 10,000 cells from each tube are typically analyzed to produce statistically valid information. (c) Each cell passes individually through the highly focused laser beam of the flow cytometer, a process termed single cell analysis. The fluorochrome of each labeled monoclonal antibody attached to the cell is excited by the laser light and emits light of a certain wavelength. The cells also scatter light at multiple angles. Photodetectors placed a forward angle and at right angles to the axis of the laser beam collect the emitted or scattered light. Forward and right angle scatter signals, and as many as five fluorochrome signals can be detected from each cell (multiparametric analysis). (d) The signals from each photodiode are digitized and passed to a computer for storage, display, and analysis. Typically, all data recorded from each cell is stored, for possible later recall for further analysis ("list mode data storage"). (e) A variety of histograms for visual display can be generated automatically or at the discretion of the operator. List mode data can also be transferred to a separate computer for analysis. Presently, most commercial flow cytometers utilize a standardized file format for list mode storage, and a variety of computer programs are commercially available for data analysis and display.

capacity to simultaneously record and analyze three or four fluorescence signals along with forward and right angle light scatter. The multiparametric capability of the flow cytometer is often needed to analyze clinical specimens, which are rarely

homogenous in nature, and most often are comprised of mixtures of normal and neoplastic cells. The process of "gating" is used to identify one or more cell populations of interest in a specimen and to electronically separate them from normal cell populations, clinically nonrelevant cell populations or contaminants. The proper gating of a neoplastic population is a subjective process that requires knowledge of the normal cell composition of the specimen under analysis and the proper combination of electronic signals. Generally, a graphic display of the

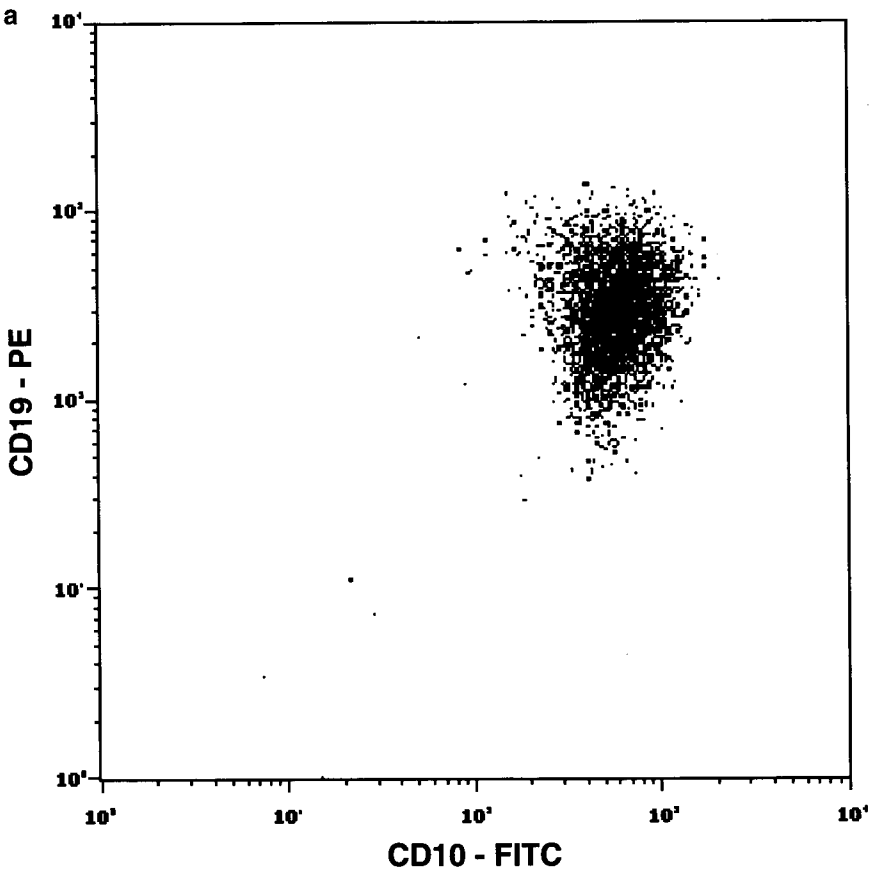

Fig. 3. Scattergram versus histogram display of flow cytometric data. The data is from a patient with childhood ALL with a B-cell phenotype. (a) The dual parametric scattergram shows the intensity of light emission from FITC-labeled anti-CD10 (x-axis, log scale) and PE-labeled anti-CD19 (y-axis, log scale). Each dot represents the data obtained from a single cell. In this case, the cells show bright, relatively uniform expression of both CD10 and CD19, generating a tightly clustered dot pattern. (b) Single channel histogram showing CD10 expression for the cells that were analyzed. The intensity of CD10 expression is shown on the x-axis (log scale), while the y-axis shows the number of cells showing each level of brightness (linear scale). In this case, the ALL cells brightly express CD10 and generate a homogenous peak.

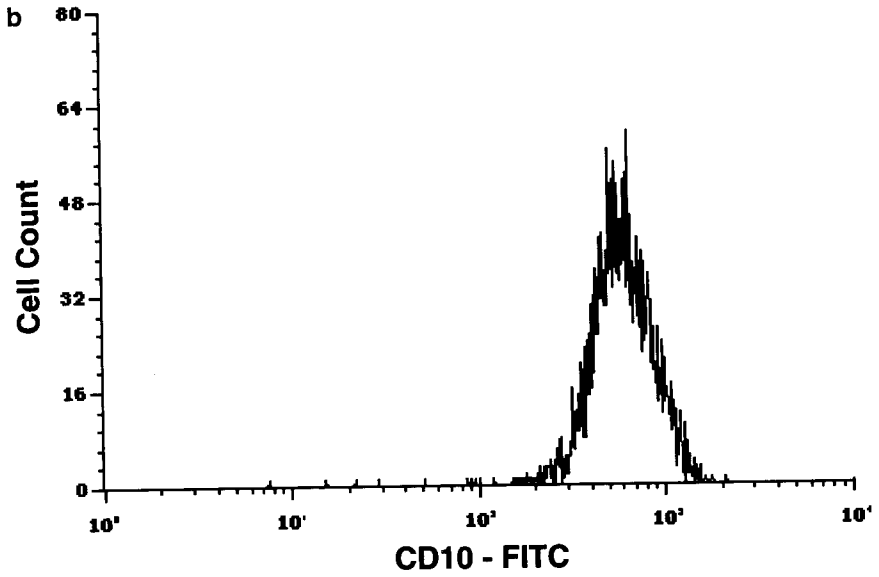

Fig. 3 (*continued*).

relevant light scatter and fluorescence signals are examined, the cell population(s) of interest are identified and their boundaries are electronically demarcated with circular, ovoid or polygonal boundaries ("bitmaps"). Then, the specimen is further analyzed to provide specific information about the cells in each gate. Electronic gating is essential for the efficiency of clinical flow cytometric analysis, since large numbers of specimens can be analyzed without the need for time consuming physical separation of cell populations prior to staining. Most flow cytometers permit the simultaneous delineation of three or more cell populations for gated analysis. Fortunately, flow cytometric data can be stored in a "list mode" format so, if necessary, one can modify the original gates and digitally reanalyze data later.

Any parameters acquired by the flow cytometer can be used as a signal for electronic gating. From the late 1970s until the 1990s, most clinical flow cytometers could acquire only two fluorescence signals in addition to forward angle and side-scatter. At that time, gating was usually performed using forward angle light scatter verses right angle light scatter for the selection of neoplastic cell populations. However, leukemic blasts and normal lymphocytes often fell into the same area of the histogram display and could not be separately gated for analysis. The availability of three-color flow cytometers and the discovery that leukemic blasts nearly always show less intense CD45 expression changed the method of gating for leukemic analysis. Stelzer and associates, and later Borowitz and collaborators, and Sun et al showed that a gating strategy combining CD45 expression versus RALS was far superior to FALS versus RALS for the identification of leukemic cell populations in peripheral blood or bone marrow specimens

[32–34]. Furthermore, CD45 was combined with multiple pairs of fluorescein isothiocyanate (FITC) and phycoerythin (PE)-conjugated monoclonal antibodies for three-color analysis of antigen expression in the specimens (Fig. 4). A recent review of this technique has been published [35].

Data analysis, interpretation, and reporting

With full knowledge of the clinical facts, and the personal assurance of adequate data collection and analysis, the flow cytometric histograms are examined and interpreted with reference to the clinical question. During this

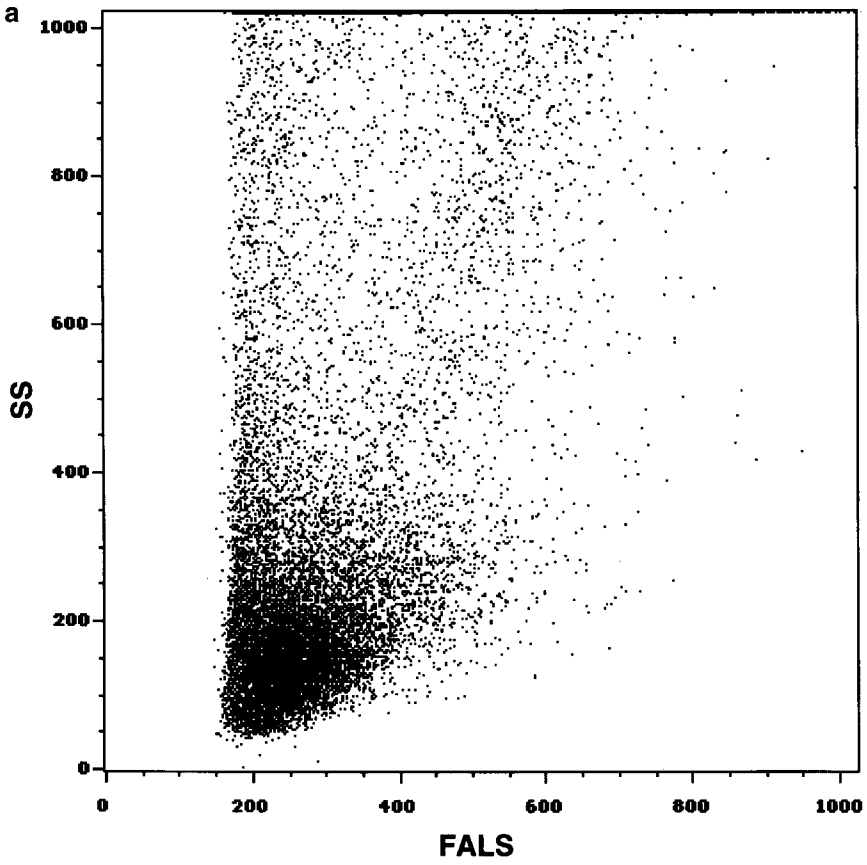

Fig. 4. Scattergrams of a bone marrow aspirate from a patient with ALL. (a) A scattergram of forward angle light scatter (FALS, x-axis) vs. side scatter (SS, y-axis) shows a relatively homogenous cell population with FALS and SS. (b) A scattergram of CD45 fluorescence intensity vs side scatter permits the discrimination of blast cells (dim CD45 expression) from mature lymphocytes (bright CD45 expression). The scattered cells with high side scatter and variable CD45 brightness are myeloid cells. (c) A polygonal gate has been drawn for gated analysis of the blast cell population.

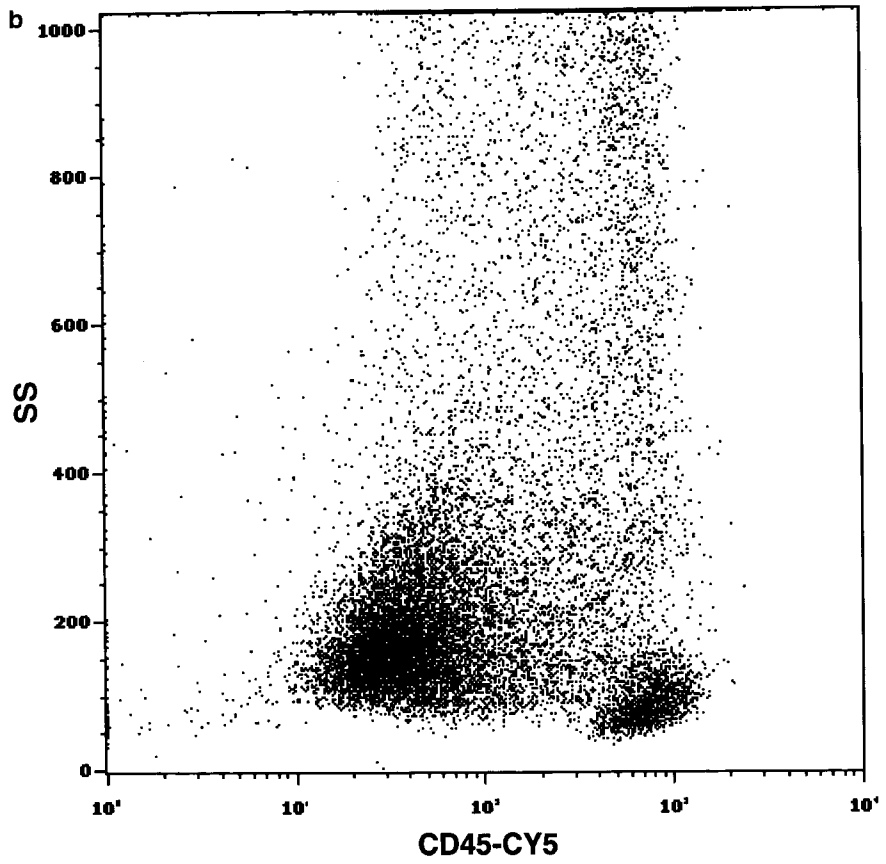

Fig. 4 (*continued*).

process, the following questions should be answered for each cell population that was identified:

- How many cell populations are present in the cell suspension? What is the relative proportion of each?
- How and to what degree do the cell populations differ from one another?
- What classification group does each cell population fit? Are the cells leukocytes? Are the cells of T-lymphoid, B-lymphoid, or myelomonocytic lineage?
- What maturation antigens are present?
- Do the light scatter properties and histological properties of the cells correlate with their immunophenotypic features?
- Are additional markers or laboratory procedures required for further delineation of the cells?

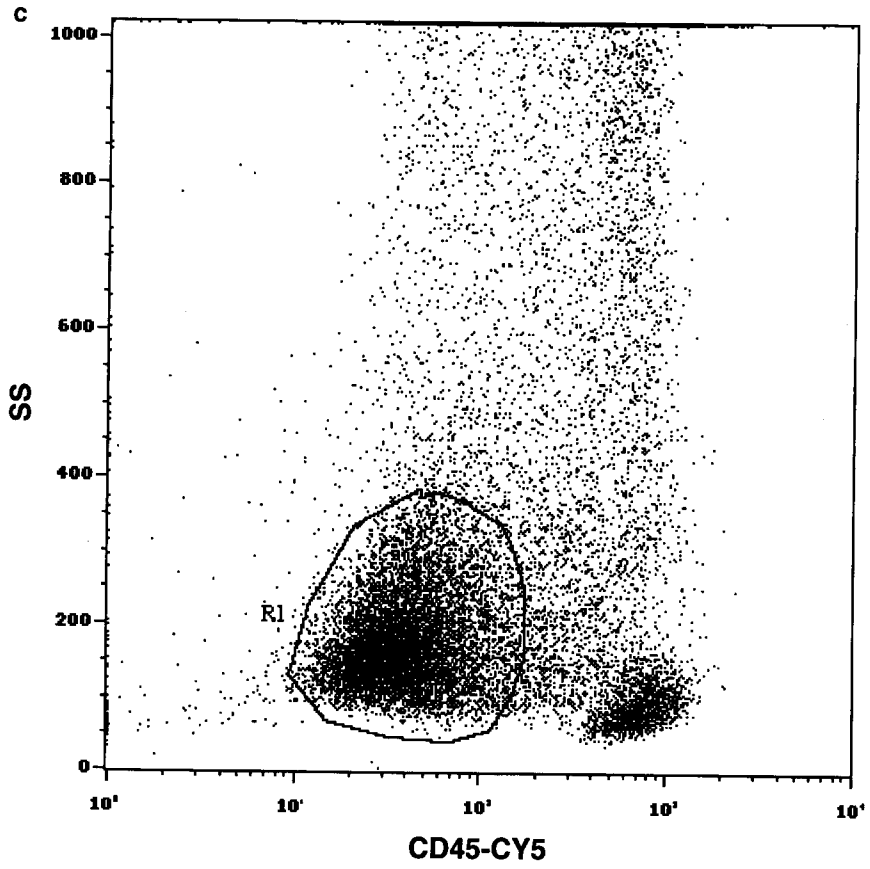

Fig. 4 (*continued*).

After the relative composition of each population is established, the data is further examined for evidence to assist in resolving the clinical question. This evidence is usually in the form of:

- Quantitative changes in populations or subpopulations
- Maturation shifts (Less mature, activated)
- Monoclonality

Diagnostic application of immunophenotypic analysis of ALL

Immunologically, lymphoblasts may express antigens common to different stages of T- or B-lymphocyte lineage; however, B-ALL is the most common subtype of ALL, and comprises 75% to 85% of ALL cases. These cases usually originate from B-lymphocytes at relatively early stages of their development. Since surface immunoglobulin is not expressed at these early stages, antibodies

against a series of B-specific antigens have been especially useful in the study and diagnosis of these leukemias.

Precursor B-Cell ALL

The diagnosis of B-ALL primarily relies on the reactivity of two monoclonal antibodies, CD19 and HLA-DR. Antibodies against the CD19 antigen have been especially helpful because CD19 is the earliest B-lineage-specific antigen presently known and it precedes the appearance of HLA-DR, CD10, CD20 and other B-specific antigens. Lack of reactivity with CD19, for all practical purposes, rules out a B-lineage of the leukemia. CD19 is present from the time of B-lineage commitment of the hematopoietic stem cell through the stages of pre-B and mature B-cell differentiation [36]. It is finally down regulated during terminal differentiation of the B-lymphocyte into the plasma cell. Since CD19 expression is maintained during B-cell neoplastic transformation, CD19 expression is useful in diagnosis of leukemias and lymphomas of B-cell lineage [36,37]. Engagement of the CD19 receptor leads to the activation of the Src family tyrosine kinase LCK (p56lck), enhanced tyrosine phosphorylation of multiple substrates, and activation of protein kinase C [38]. CD79a is useful in the rare B-cell malignancy with equivocal CD19 expression [5,39]. Intense HLA-DR reactivity is very commonly seen in B-ALL, most acute myelocytic leukemias and in a significant minority of T-cell malignancies. For this reason, a negative reaction with HLA-DR is more informative than a positive one in the diagnosis of B-ALL. The other monoclonal antibodies against the B-cell related surface markers (such as CD10, CD20, CD21, CD22, CD24, CD79a, cytoplasmic immunoglobulins, and surface immunoglobulins) are useful in confirming B-lineage and in subclassification of the leukemia. Equally important in the diagnosis of B-ALL is a lack of reactivity with T-lymphoid and myeloid cell surface markers. The use of monoclonal antibodies to all three main cell lines (B-lymphoid, T-lymphoid, and myeloid) in the evaluation of all acute leukemias is critical in view of the frequently reported cases of acute leukemia that demonstrate surface markers from more than a single cell line (Fig. 5).

The CD10 (cALLA) antigen, found commonly in childhood B-cell leukemia, is an especially useful marker of leukemic cells in blood or CSF, since it is present only on a very small fraction of normal cells. By multiparametric analysis, approximately one cALLA+ cell among 100,000 normal lymphocytes can be detected, providing a sensitive technique for the early detection of most childhood leukemias. The level of CD10 expression is of prognostic significance, since it correlates with chromosomal abnormalities. High CD10 levels ($> 3 \times 10^4$/cell) are characteristic of hyperdiploidy, low CD10 levels ($1.8–4 \times 10^3$/cell) correlate with a translocations between chromosomes 1 and 19 [t(1;19)], and undetectable CD10 levels ($<1.2 \times 10^3$/cell) are common in ALL patients with a translocation between chromosomes 4 and 11 [t(4;11)(q21;q23)] translocation [40]. Rearrangement of the IGH genes and the expression of CD15 are also characteristic of CD10− (PreB1) ALL [41]. Aberrant overexpression of CD10 is found in nearly 44% of ALL cases, and is maintained during disease relapse [40]. Children with precursor B-ALL and

the common t(12;21)(p13;q22) fusion gene (ETV6/AML1) show more intense expression of the CD10 and HLA-DR antigens, and less intense expression of the CD20, CD45, CD13, and CD34 antigens than t(12;21)$^-$ patients [42].

Adult patients with B-ALL and the BCR/ABL gene rearrangements [ie, Philadephia chromosome, t(9;22)(q34;q11.2)] show a unique immunotphenotype characterized by relatively bright and homogenous CD10 and CD34 expression, aberrant CD13 expression, and dim, heterogenous expression of CD38 [43]. Table 2 summarizes many of the known correlations between immunophenotypic characteristics and chromosomal abnormalities.

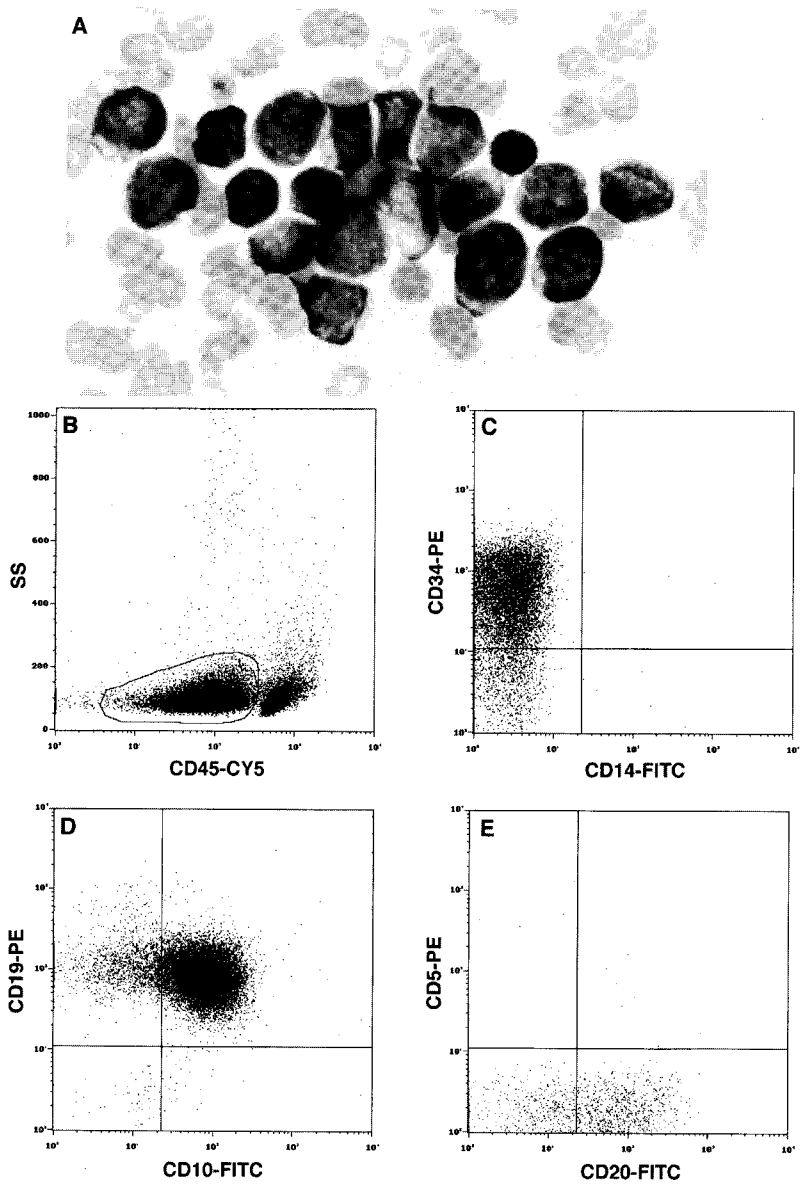

Fig. 5. Immunophenotypic analysis of a three-year-old female with ALL of B-cell lineage. (A) Photomicrograph of the bone marrow showing a cluster of blast cells with fine chromatin texture, inconspicuous nucleoli, and small amounts of cytoplasm. The blasts show FAB-L1 morphology by the FAB classification scheme. (B) A histogram of CD45 expression versus side scatter showing a polygonal gate. Approximately 76% of the total cell population showed the dim CD45 expression characteristic of blasts and were included in the gate. (C) A scattergram of CD14 (x-axis) and CD34 expression (y-axis). The majority of the cells (83%) showed moderately bright CD34 expression, but CD14 is negative. (D) A scattergram of CD10 (x-axis) and CD19 expression (y-axis) showing bright dual expression of these antigens by more than 90% of the gated cells. (E) A scattergram of CD20 (x-axis) and CD5 expression (y-axis) showing heterogenous CD20 expression and negative staining for CD5 by the gated cells.

Flow cytometric determination of terminal deoxynucleotidyl transferase (TdT) has also proven useful in the differentiation of reactive and lymphoblastic cells. Unfortunately, TdT is technically more difficult to analyze than cell surface antigens because of its intranuclear location. Presently, TdT analysis requires cellular permeabilization with diethylene glycol-based red blood cell lysing solution, saponin, detergent, methanol, or other agents prior to staining with fluorochrome-labeled anti-TdT monoclonal antibodies [44–48]. Under these circumstances, flow cytometric analysis of TdT was found to be rapid, reproducible, objective and reliable in clinical practice. In a comparative study with other methods, TdT analysis by flow cytometry was found to be 100% concordant with the results obtained by the biochemical TdT assay, immunoperoxidase determination and fluorescence microscopy [49]. Rare cases of T-ALL are negative for TdT.

Precursor T-Cell ALL

Leukemias of T-lineage (T-ALL) comprise 15% to 25% of ALL cases. Clinically, most patients are older males who present with high peripheral blast counts and mediastinal masses. Flow cytometric diagnosis of T-ALL is more difficult than that of B-ALL for the following reasons:

- Demonstration of "monoclonality" is not as comfortably achieved in T-ALL as it is in B-ALL where the cells could carry a single immunoglobulin light chain kappa or lambda.
- Markers that are detected only in the early phases of T-cell maturation and are absent in mature T cells (eg, CD1b) are few and occur uncommonly in T-ALL as compared to the relatively more common occurrence of similar markers (eg, CD10 or cytoplasmic immunoglobulins) in B-ALL.
- HLA-DR is commonly absent in T-ALL but its occurrence in B-ALL is very helpful because its reaction with anti-DR antibody is much more intense in immature cells than in mature B-cells.

Table 2
Correlations between immunophenotype characteristics and chromosomal abnormalities

Chrosomal abnormality	Phenotypic characteristics	Oncogene	Frequency	Clinical features
Hyperdiploid karyotype	B-lineage immunophenotype. Bright CD10 expression, grooved or radially segmented blast nuclei (Rieder's cells).	—	2–9% adult ALL patients.	Younger age at diagnosis. Normal white blood count.
Hypodiploid karyotype	Common ALL immunophenotype with either FAB-L1 or FAB-L2 morphology.	—	4–9% adult patients	Younger patients, unfavorable prognosis
Near tetraploid karyotype	Precursor T-cell phenotype, L2 morphology, expression of myeloid-associated antigens (CD13, CD15, CD33).	—		Older age at diagnosis
Normal karyotype	T-lineage immunophenotype in 18–38%	None	15–34% adults	Intermediate age and white blood count. Variable prognosis.
Tetraploid karyotype	Frequent T-lineage immunophenotype.	—		Relatively good prognosis.
del(6p), del 6(q)	Frequent T-lineage immunophenotype.		5–10% of chromosomally abnormal ALL cases, sole change in two-thirds	Relatively good prognosis.
t(1;14)(p32;q11)	T-ALL T-ALL, tald rearrangement. CD1-CD2 + CD4-CD7 + CD10- phenotype (Stage 1 thymic differentiation).	TAL-1/TCR	20%	Good prognosis
t(1;19)(q23;p13)	Pre-B ALL. FAB L1/L2 phenotype. TdT + CD9 + CD10+(dim)CD19 + CD22 + HLA-DR+. Absence of CD20, CD34, and myeloid-associated antigens. Cytoplasmic Ig mu expressed in most cases (C mu+).	E2A/PBX1 fusion transcript in C mu+ cases	25% children, 3% adults	Young age, low white blood counts, unfavorable prognosis.
t(2;16)(p11.2;p11.2)	B-lineage ALL. FAB-L1 phenotype. Low hyperdiploidy (47–49 chromosomes) with structural abnormalities.	?		First noted in childhood ALL. Variable WBC, high incidence of relapse, poor prognosis
t(4;11)(q21;q23)	Pre-B immunophenotype (CD19 + CD24dimCD10-CD20-/cylgM-sIgM-). Expression of myeloid-associated antigens (CD15, CDw65+). IgH rearrangement.	AF4/MLL	> 10% ALL (50% infants, 3–7% adults).	Mostly female patients. Very high white blood count, CNS involvement, and very poor outcome.
t(4;11)(q21;p14-15)	FAB-L1 morphology. CD2, CD5, CD7, CD10 (CALLA), CD34, and HLA-DR. Frequent expression of CD33. Rearrangement of immunoglobulin heavy- chain genes.	?		
t(8;14)(q11.2;q32)	CD10+ B-precursor phenotype	?	Rare	Children
t(8;14)(q24;q32) t(8;14)(q24;q11) t(2;8)(p12;q24)	Mature B-ALL, FAB-L3 phenotype	C-MYC/IgK	100% (ALL, FAB-M3), 5% all ALLs	
t(9;22)(q34;q11.2)	FAB-L2 morphology. Precursor B-cell phenotype [TdT + CD10+ CD34 + CD38+ (dim)] with expression of myeloid-associated antigens. Deletion of chromosome 7 in 25% of childhood ALL with t(9;22).	BCR/ABL	5% children, 10–30% adults	Older patients with high white blood count at presentation. Very poor prognosis.
t(10;14)(q24;q11), other t(14q11-q13) translocations	Frequent T-lineage immunophenotype.	NOX11/TCR	4–6% adult ALL patients.	Favorable outcome
t(11;14)(p13;q11)	T-ALL	RHOM-2/TTG2	10%	

(continued on next page)

Table 2 (*continued*)

Chrosomal abnormality	Phenotypic characteristics	Oncogene	Frequency	Clinical features
11q23 translocations [t(4;11). t(1;11), t(11;19)] t(11;19)(q23;p13)	Early B-cell ALL Pre-B immunophenotype (CD19+ and Tdt+) with expression of CD13 and CD33. Heavy chain immunoglobulin gene rearrangement. Mixed myeloid/lymphoid lineage.	ALL-1 (HRX) MLL	5% adults, 70% infants (< 18 months) Mostly in male infants.	Extremely poor prognosis "Hand-mirror" morphology, predominately female patients with an indolent course
t(12;21)(p13;q22)+	Bright CD10 and HLA-DR expression and relatively dim expression of CD20, CD45, CD135, and CD34. Heterogenous CD34 expression. Lack of CD9 expression	TEL/AML-1	> 20% of precursor B-ALL.	Excellent prognosis.
t(14;18)(q32;q21)	Pre-B phenotype without expression of cytoplasmic or membranous Ig. Frequent complex chromosomal and molecular abnormalities involving at least the BCL-2 and c-MYC genes. Frequent chromosomal abnormalities, including translocation t(8;14) and deletion of chromosome 9.	Ig heavy chain (IgH)/BCL-2	Rare ALL patients. 80% of adults with non-Hodgkin's lymphoma of follicular center cell origin.	Acute clinical presentation. Nodal and/or extranodal disease, massive bone marrow infiltration and rapid increase of circulating blast cells of mature B cell phenotype. High incidence of neuro-meningeal relapse. Very poor prognosis.

- It is difficult to demonstrate distribution shifts in T-ALL because, T cells are the dominant type of lymphocyte in most normal specimens. An exception is the lymph node, where T lymphocytes comprise about 60% of the total cell population.
- The presence of almost a single subtype of T cell (CD4 or CD8) can be seen in a number of non-malignant conditions.

There are several combinations of T-cell and T-cell related lymphocytic cell surface markers that assign a T-cell lineage with varying degrees of certainty (Fig. 6). As with all other immunophenotypic analysis, the clinical as well as other investigational findings are an extremely important component of the final diagnosis. Some of these combinations are described below:

- An almost unique population of cells showing positive reactions with CD7, CD1a, CD3 and/or CD2, with cells showing simultaneous positivity with CD4 and CD8, and with negative reactions to B-lymphocytic and myelomonocytic cell surface markers is diagnostic of acute T-ALL in any tissue except in thymus where the thymocytes normally express CD1a and dual marking with CD4 and CD8 during their maturation.
- An almost unique population of cells showing positive reactions with CD7, CD3 and/or CD2, and negative reactions with CD4 and CD8, as well as with B-lymphocytic and myelomonocytic cell surface markers also is diagnostic of acute T-ALL in any tissue, including in thymus.
- An almost unique population of cells showing positive reactions with CD7, CD3 and/or CD2, CD4, or CD8, and with negative reactions with B-lymphocytic and myelomonocytic cell surface markers is strongly suggestive of acute T-ALL in any tissue, including the thymus. Since a CD4 or CD8 phenotype can be a very dominant feature in many other situations, this combination of markers should be evaluated with great caution. A marked increase in CD8 lymphocytes to the point of outnumbering the CD4 positive cells is less commonly seen than a CD4 domination because the CD8 positive T cells are normally much less numerous than the CD4 positive cells. Marked CD8 domination over CD4 positive cells can be seen in HIV infections, especially with superimposed opportunistic infections, and in some other viral infections. Marked CD8 domination over CD4 positive cells can be seen in Hodgkin's disease and several autoimmune disorders.
- An almost unique population of cells showing positive reactions with only CD7 and with negative reactivity with CD3, CD2, CD4 and CD8 as well as with B-lymphocytic and myelomonocytic cell surface markers is diagnostic of T-ALL provided sufficient number of B-lymphocytic and myelomonocytic cell surface markers have been used to rule out the presence of other types of leukemias that display CD7.

The most sensitive marker for T-ALL appears to be the pan-T 40 kd antigen defined by anti-Leu-9 (CD7). This marker has been detected in T-ALL with

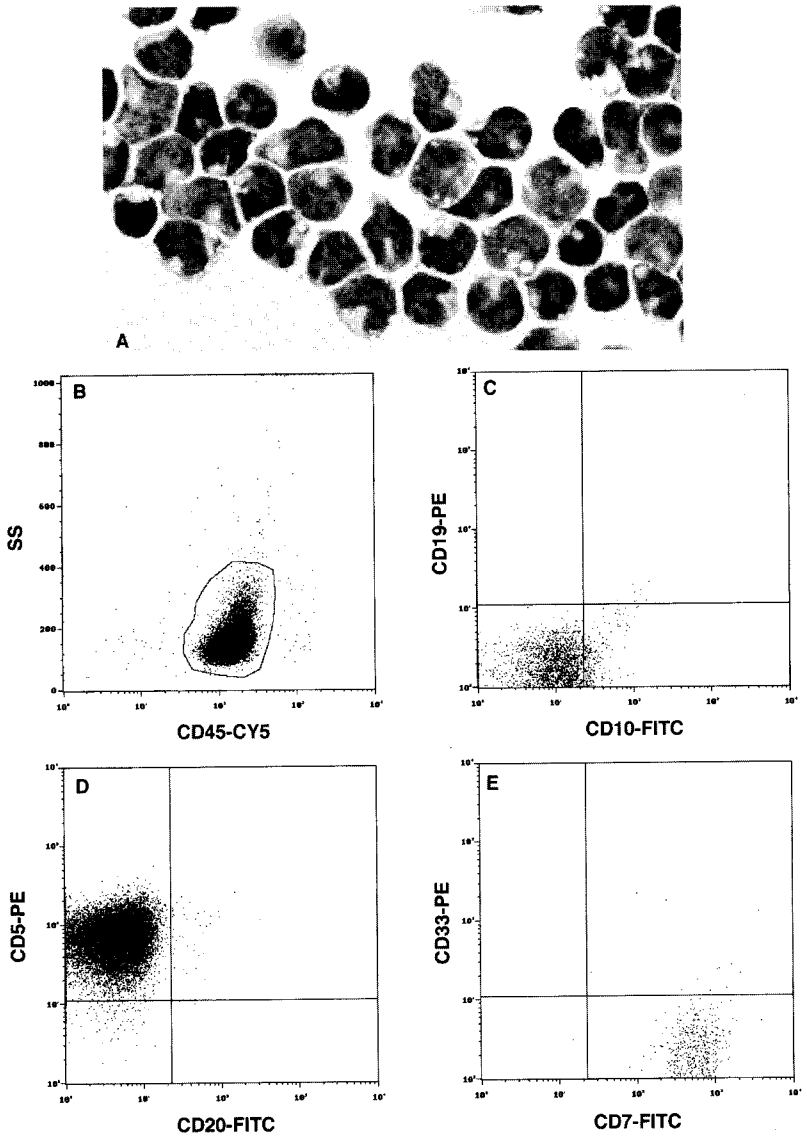

Fig. 6. Immunophenotypic analysis of a 22-year-old male with ALL of T-cell lineage. (A) Photomicrograph of the bone marrow showing a cluster of blast cells with fine chromatin texture, inconspicuous nucleoli, and small amounts of cytoplasm. (B) A histogram of CD45 expression versus side scatter showing a polygonal gate enclosing the CD45-dim blast cells. Approximately 99% of the total cell population showed the dim CD45 expression characteristic of blasts and were included in the gate. (C) A dual parameter scattergram of CD10 (x-axis) and CD19 expression (y-axis) showing a lack of expression of either antigen by the gated cells. (D) A scattergram of CD20 (x-axis) and CD5 expression (y-axis) showing moderately bright relatively homogenous CD5 expression and negative staining for CD20 by the gated cells. (E) A scattergram of CD7 (x-axis) and CD33 expression (y-axis) showing moderately bright relatively homogenous CD7 expression and negative staining for CD33 by the gated cells.

frequencies of greater than 95% to 100% [50–52]. Because of this high incidence of CD7 positivity in T-ALL, a diagnosis of this type of leukemia should not be made in the absence of CD7 positive cells. On the other hand, the presence of CD7 positive blasts is by no means synonymous with a diagnosis of T-ALL, because a large number of acute leukemias of B-lymphocytic and myelocytic lineage have been associated with the expression of CD7 on the cell surface. Zutter et al found as many as 26% of patients with acute myelocytic leukemias to express CD7 [53]. Other authors have also reported a significant percentage of acute leukemias that demonstrate CD7 (often with other T-cell markers) with either intralineage or interlineage pattern. These observations emphasize the following concepts in the diagnostic approach to T-ALL:

- A diagnosis of T-ALL should be made with great caution in the absence of reactivity with CD7.
- A diagnosis of T-ALL should generally be made when other T cell markers (eg, CD2, CD3, CD5) are also present in addition to the CD7 antigen.
- CD5 and CD2 are expressed in most cases of T-ALL.

Although this classification attempts to represent various levels of T-cell maturation, Roper et al found no unique clinical features among the subgroups and no difference in remission duration or survival [54].

Rare leukemias and lymphomas are derived from large granular cell/natural killer cell (LGL/NK) precursors. The nomenclature of these neoplasms is less than adequate, but they have been considered a subset of the LGL lymphoproliferative diseases, which include T-LGL leukemia (CD3+), NK-LGL leukemia (CD3−), and LGL lymphocytosis (CD3− or +). Clinically NK-LGL leukemias are aggressive neoplasms usually found in adults with extranodal involvement. L2 lymphoblast-like morphology is present, with variable cell size, round to moderately irregular nuclei, prominent nucleoli, and pale cytoplasm without granules [55–57]. The usual phenotype is CD3−CD7±cytoplasmic CD3±CD16−CD33+CD34+CD45−CD56+MPO−HLA-DR±TdT−. Germline configurations of the T-cell receptor beta and gamma chain genes and Ig heavy chain gene are usually present. Scott et al isolated a punitive precursor cell common to myeloid and NK-cell lineages, and proposed the term myeloid/natural killer cell acute leukemia for these neoplasms [58]. In infants, the blastic cytologic features of the blast cells can be mistaken for conventional small, round, blue cell tumors [59]. NK-LGL leukemias have been reported to arise in patients with altered immune systems, including those undergoing solid organ transplantation and those with chronic myelogenous leukemia, non-Hodgkin's lymphoma, essential thrombocythemia, and midline lethal granuloma [60–66]. T-LGL leukemias are rare neoplasms with monocytic features, which have been mistaken for acute monocytic leukemia or hairy cell leukemia. The characteristic immunophenotype is CD2+CD3+CD4CD8±CD11b+CD45+CD56+CD38+ [67,68]. Most of these tumors appear to have a relatively benign clinical course, but aggressive forms have been described [67].