



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 classified 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 non-morphologic data, including flow cytometry, and is followed in this article (Fig. 1) [10].

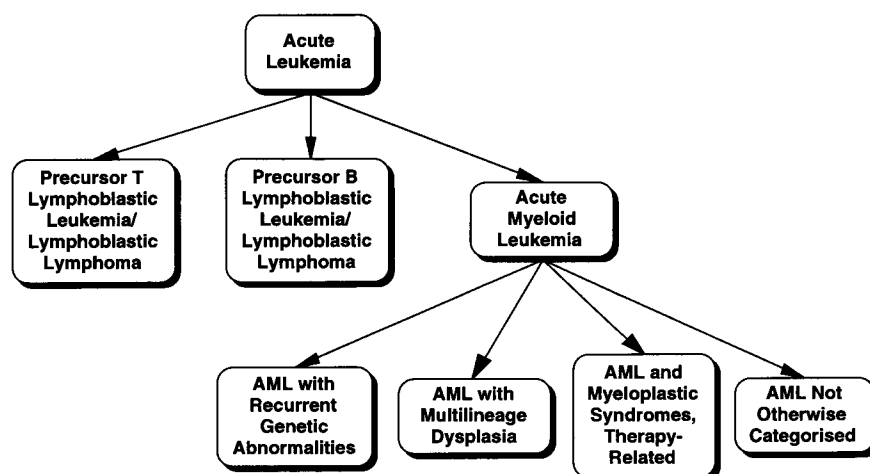


Fig. 1. World Health Organization classification of acute leukemia, 2001.

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

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/ μ L.

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, ethylenediamine-tetraacetic 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 fluoro-chrome-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 fluoro-chrome-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	Szary cell leukemia and some hematopoietic neoplasms of T-cell lineage.	Coreceptor for MHC class II molecules. Binds lock on cytoplasmic face of membrane.	T4, Leu-3a
CD5	T cells, thymocytes, B cell subset	B-CLL and most hematopoietic neoplasms of T-cell lineage.	Receptor for HIV-1 and HIV-2 gp120. 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-cell activation. 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 lock 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.	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

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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 γ 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. Leukocyte activation.	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.		Leu17, T10

CD41	Megakaryocytes, platelets	Acute leukemia of megakaryocytic lineage (AML, FAB-M7).	allb integrin. Associates with CD61 to form GPIIb. Binds fibrinogen, fibronectin, von Willebrand factor and thrombospondin	GPIIb/IIIa, PL-273
CD42b	Megakaryocytes, platelets	Acute leukemia of megakaryocytic origin (AML, FAB-M7).	GPIb, vWF receptor. Binds von Willebrand factor and thrombin.	GPIb, FMC-25
CD43	T cells, myeloid cells, some B cell lymphomas	Some T-cell lymphoproliferative diseases.	Essential for platelet adhesion.	Leu22
CD45	Panhematopoietic	All hematopoietic neoplasms.	Leukosialin, sialophorin binds CD54 (ICAM-1).	T200, LCA
CD45RA	B cells, T-cell subsets (naive T-cells) monocytes		Leukocyte common antigen. Tyrosine phosphatase, augments signalling through antigen receptor of B- and T-cells.	2H4
CD45RO	T-cell subsets, B-cell subsets, monocytes, macrophages		Isoforms of CD45 containing the A exon.	UCHL-1
CD55	Widespread cellular distribution, hematopoietic and non-hematopoietic cells	Absent or deficient in paroxysmal nocturnal hemoglobinuria (PNH).	isoform of CD45 containing none of the A, B and C exons.	DAF
CD56	NK cells	Hematopoietic neoplasms of NK-cell lineage.	Decay Accelerating Factor (DAF). Complement activation inhibitor. Binds C3b, disassembles C3/C5 convertase	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.	NKH-1. Cell adhesion molecule. Isoform of Neural Cell Adhesion Molecule (NCAM).	HNK-1, Leu7
CD59	Many hematopoietic cells	Absent or deficient in paroxysmal nocturnal hemoglobinuria (PNH).	many cell surface glycoproteins. Protectin, Mac inhibitor. Complement inhibitor. Binds complement 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).	Pt GPIIa, 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>.

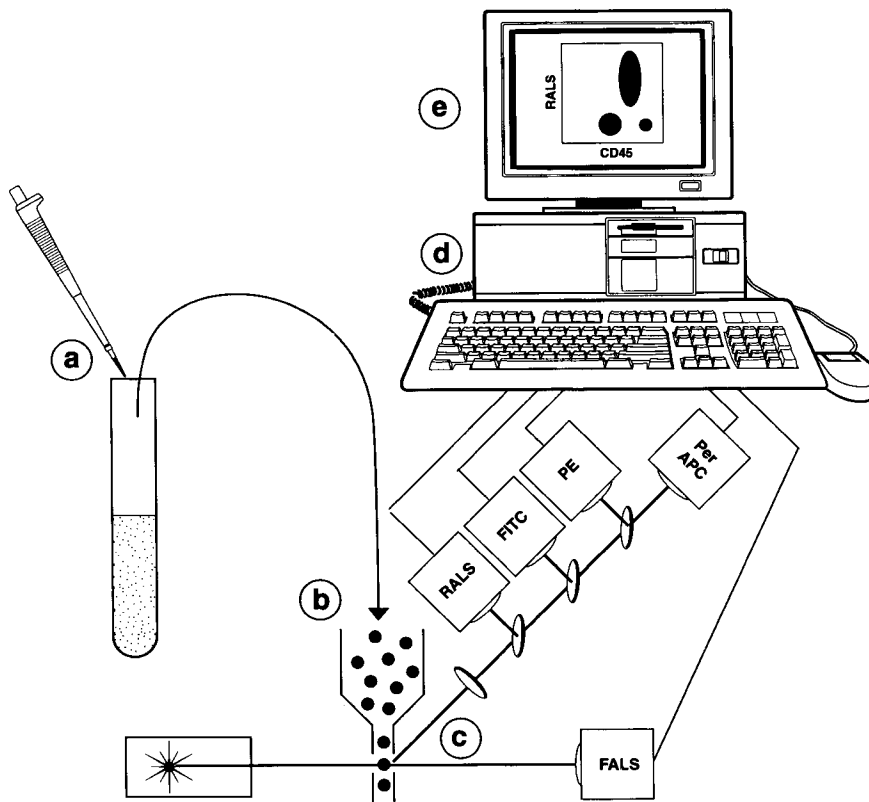


Fig. 2. Immunophenotypic analysis of an acute leukemia by flow cytometry. (a) Fluorochrome-labeled monoclonal antibody solutions are added to a cell suspension from a peripheral blood, bone marrow aspirate, or lymph node. The tubes are incubated at room temperature for a short period. (b) The labeled 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 at 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

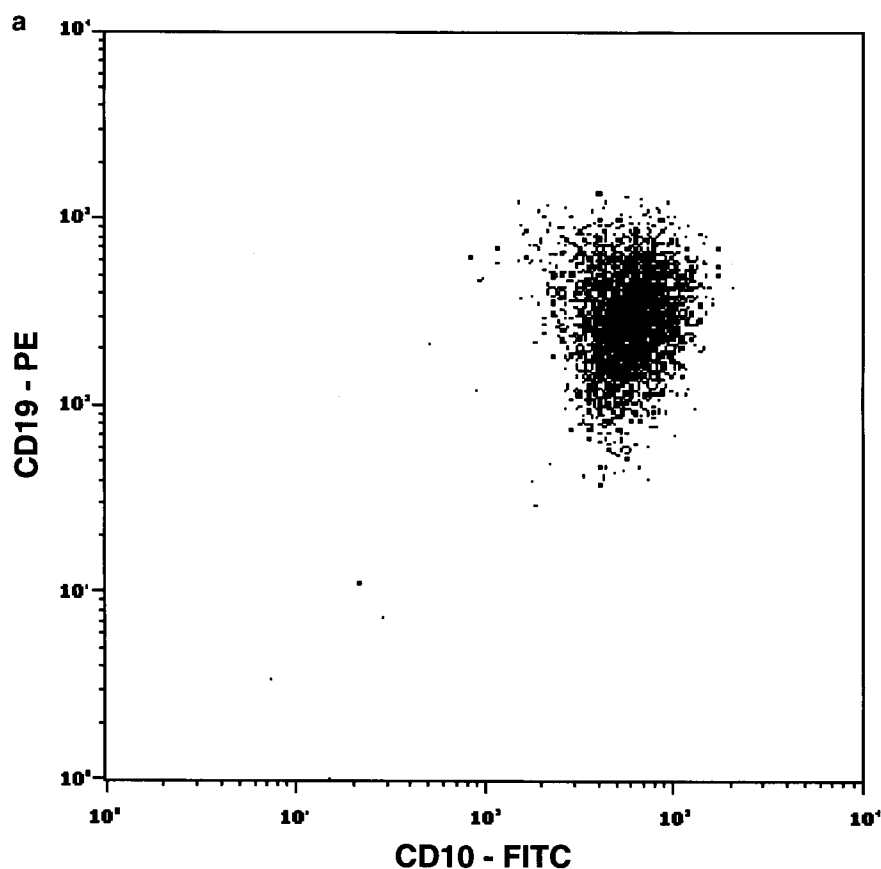


Fig. 3. Scattergram versus histogram display of flow cytometric data. The data is from a patient with childhood ALL with a B-cell phenotype. (a) The dual parametric scattergram shows the intensity of light emission from FITC-labeled anti-CD10 (x-axis, log scale) and PE-labeled anti-CD19 (y-axis, log 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.

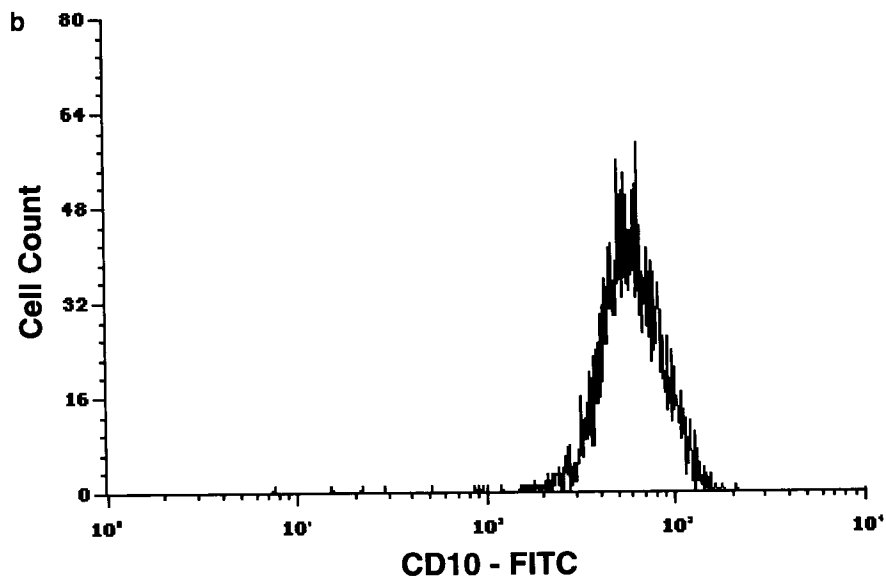


Fig. 3 (continued).

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 versus 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 phycoerythrin (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

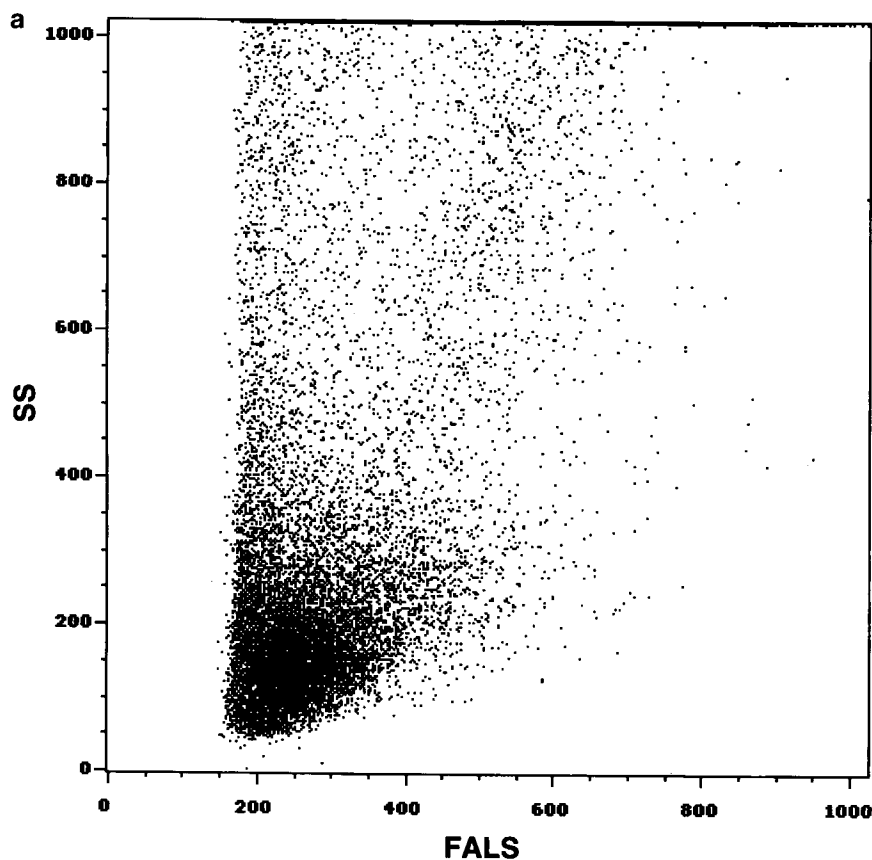


Fig. 4. Scattergrams of a bone marrow aspirate from a patient with ALL. (a) A scattergram of forward angle light scatter (FALS, x-axis) vs. side scatter (SS, y-axis) shows a relatively homogenous cell population with FALS and SS. (b) A scattergram of CD45 fluorescence intensity vs side scatter permits 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.

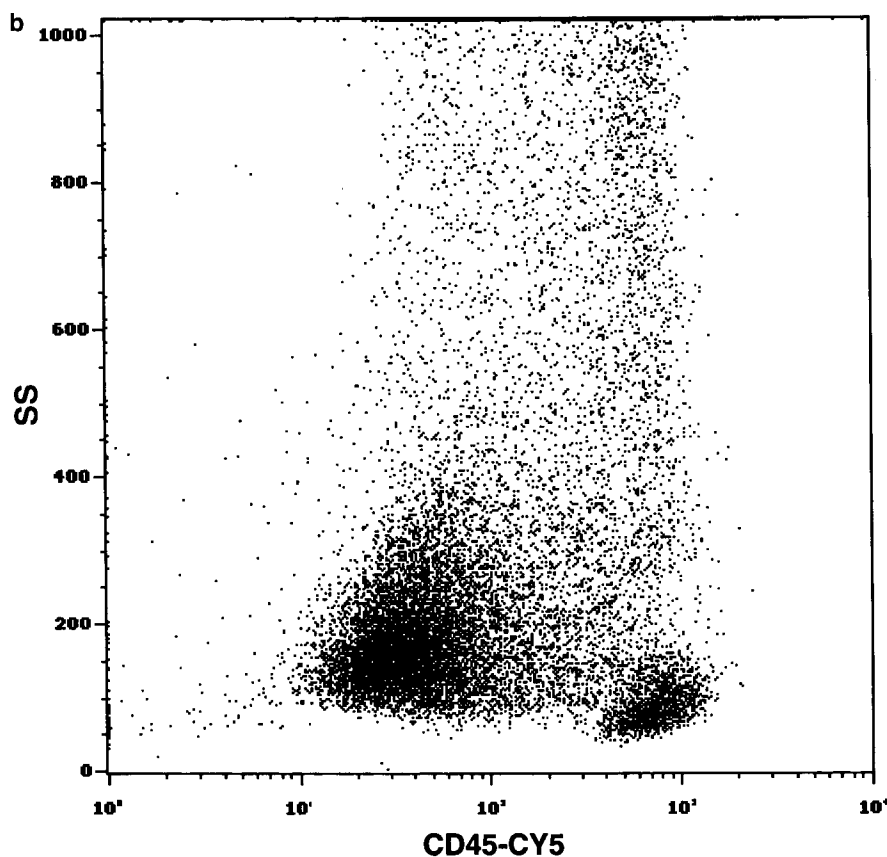


Fig. 4 (continued).

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?

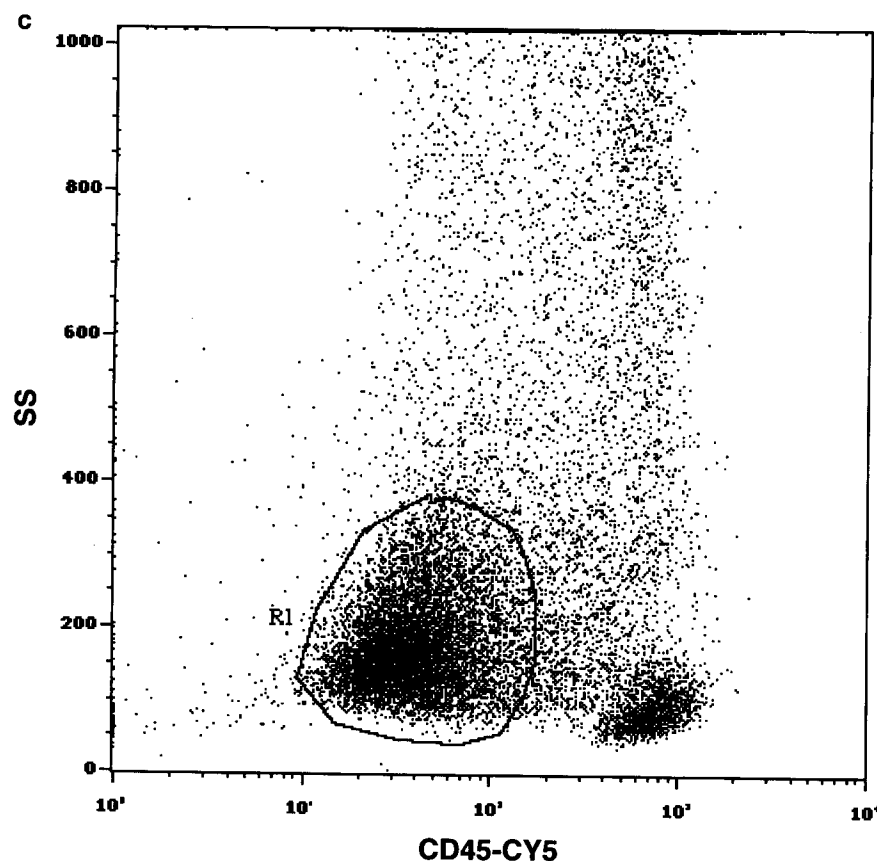


Fig. 4 (continued).

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.

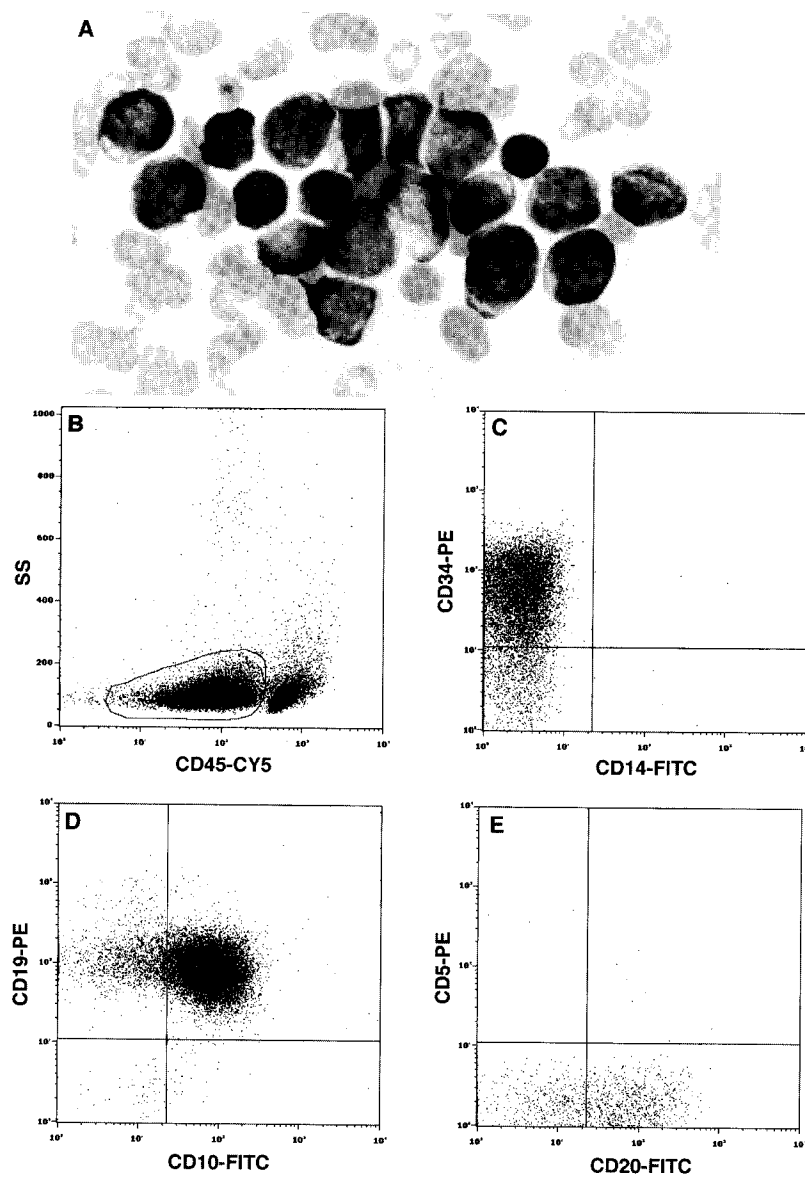
Precursor B-Cell ALL

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 ($> 3 \times 10^4/\text{cell}$) are characteristic of hyperdiploidy, low CD10 levels ($1.8\text{--}4 \times 10^3/\text{cell}$) correlate with a translocations between chromosomes 1 and 19 [t(1;19)], and undetectable CD10 levels ($< 1.2 \times 10^3/\text{cell}$) 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, Philadelphia chromosome, t(9;22)(q34;q11.2)] show a unique immunotphenotype characterized by relatively bright and homogenous CD10 and CD34 expres-



sion, aberrant CD13 expression, and dim, heterogenous expression of CD38 [43]. Table 2 summarizes many of the known correlations between immunophenotypic characteristics and chromosomal abnormalities.

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.

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.

Table 2
Correlations between immunophenotype characteristics and chromosomal abnormalities

Chromosomal 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. Relatively good prognosis.
Tetraploid karyotype	Frequent T-lineage immunophenotype.	—		
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-/cyIgM-slgM-). 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)	Mature B-ALL, FAB-L3 phenotype	C-MYC/IgK	100% (ALL, FAB-M3), 5% all ALLs	
t(8;14)(q24;q11)				
t(2;8)(p12;q24)				
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)

Chromosomal abnormality	Phenotypic characteristics	Oncogene	Frequency	Clinical features
11q23 translocations [t(4;11), t(1;11), t(11;19)]	Early B-cell ALL	ALL-1 (HRX)	5% adults, 70% infants (<18 months)	Extremely poor prognosis
t(11;19)(q23;p13)	Pre-B immunophenotype (CD19+ and Tdt+) with expression of CD13 and CD33. Heavy chain immunoglobulin gene rearrangement. Mixed myeloid/lymphoid lineage.	MLL	Mostly in male infants.	"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

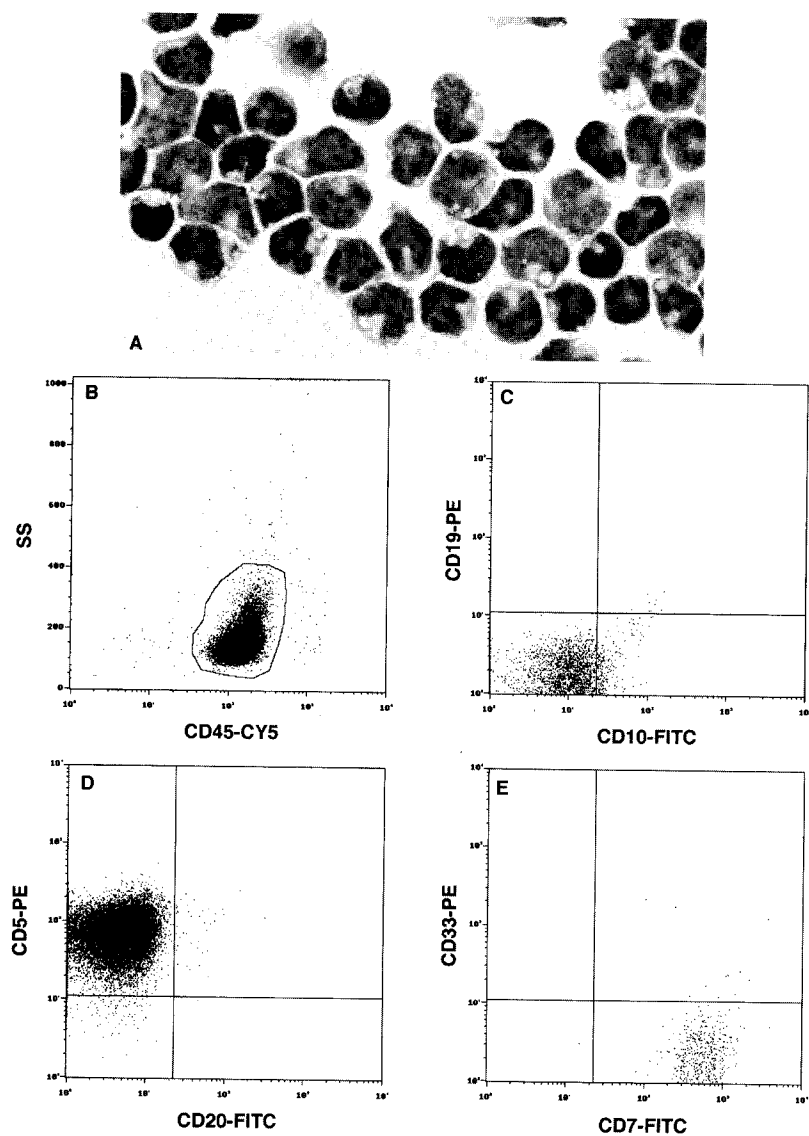


Fig. 6. Immunophenotypic analysis of a 22-year-old male with ALL of T-cell lineage. (A) Photomicrograph of the bone marrow showing a cluster of blast cells with fine chromatin texture, inconspicuous nucleoli, and small amounts of cytoplasm. (B) A histogram of CD45 expression versus side scatter showing 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 intralinear or interlinear 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 putative 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].

Diagnostic problems in immunophenotypic analysis

The vast majority of childhood and adult leukemias can be clearly assigned a lymphoid or non-lymphoid lineage by immunophenotypic analysis. However, the situation is more complex in cases of morphologically difficult or undifferentiated leukemias, when “unusual” patterns of phenotypic expression are found, when the results of immunophenotype do not correlate with other data, or in cases of disease relapse. The differentiation between the major variants of precursor-B- and T-ALL can also be a difficult problem. Under these circumstances, correlation of the immunophenotypic results with morphology and other clinicopathologic and laboratory features, and careful consideration of the relative significance of immunophenotypic evidence, usually permits the correct lineage to be identified.

Correlation of FAB morphology and immunophenotype

A common misconception about clinical flow cytometry is that an absolute “correlation” exists between morphology and immunophenotype, so that immunophenotypic analysis is often requested with the expectation of resolving ambiguities in the morphologic interpretation (ie, AML, FAB-M4 versus -M5, AML, FAB-M1 versus -M2, etc.). Unfortunately, there is no absolute correlation between surface antigen expression and morphologic appearance [69]. The closest correlations arise in the L3, M3, M6, and M7 leukemias, which have distinctive morphology and phenotype. The remaining FAB categories show characteristic immunophenotypes but ALL, FAB-L1 cannot be differentiated from ALL, FAB-L2 by immunophenotype, nor can an AML, FAB-M1 from an FAB-M2, or an AML, FAB-M4 from an M5. The subject is further complicated by the presence of aberrant antigen expression (see below). These facts should be provided to physicians who have unrealistic expectations from immunophenotypic analysis. Cytogenetic analysis may be helpful in these cases.

A few investigators have specifically examined the diagnostic effectiveness of flow cytometric immunophenotypic analysis in leukemia diagnosis. These studies revealed that ALLs can be successfully differentiated from ANLLs by morphology alone in 55% to 70% of cases, while an accurate lineage assignment could be determined in >92% of cases by the addition of immunophenotypic analysis [70–73]. Cytogenetic analysis, ultrastructural myeloperoxidase and platelet peroxidase analyses were necessary for definitive diagnosis in a few cases. Cytogenetic information contributed essential information, but it did not affect immediate diagnosis or treatment. Surprisingly, the gene rearrangement studies did not yield essential data in any case and in a few cases contributed equivocal data. Another study of interobserver variability in leukemia diagnosis revealed a concordance between observers of 63% for Wright-stained preparations alone, 89% for morphology and cytochemical stains, and 99% for light microscopy, cytochemical stains, and immunophenotypic data [72–75].

Several new classification systems of acute leukemia have been proposed that actively incorporate immunophenotypic data. For example, the French Groupe

d'Etude Immunologique des Leucemies (GEIL) proposed a scoring system in which individual markers in the B-, T- and myeloid-lineages are assigned weights of 1.5, 1.0, or 0.5 points. Lineage assignment requires a score of at least two and additional differentiation criteria are applied to T- and B-ALLs [69,76].

Aberrant antigen expression

Recently, as immunophenotypic analysis has been routinely applied to the diagnosis of acute leukemias, "aberrant" cases have been reported which do not fit into a normal immunologic classification scheme of leukocyte development, or do not correspond to known stages of normal lymphoid development. Thus, aberrant ("variant", "unusual") antigen expression is the unexpected (but reproducible and technically valid) expression of an antigen on a single leukemia cell line, the lack of expression of an expected antigen, or the presence of two or more distinct leukemia clones with different phenotypes [37,77]. These include intralinear aberrance, myeloid antigen expression in T- and B-cell leukemias, T-cell antigen expression in leukemias of B-cell lineage, lymphoid antigen expression in non-lymphoid leukemias, etc. Generally, $\geq 20\%$ of lymphoblasts must express the "unexpected" antigen under consideration to be deemed significant. Some flow cytometric studies using large panels of monoclonal antibodies have revealed that the majority of patients with ALL have at least one incidence of aberrant antigen expression, and as many as two-thirds have two or more aberrant phenotypes. Other studies have revealed a much lower incidence of aberrant antigen expression, probably reflecting differences in immunophenotypic technique, monoclonal antibody specificity, and analytic methods among different laboratories [78,79]. For example, CD45 blast gating was reported to show a higher incidence of aberrant antigen expression than the conventional forward scatter/side scatter gate [80]. Ross and collaborators analyzed 39 adult ALLs using a panel of 21 moAbs and found only two examples of cross-lineage antigen expression.

However, intralinear aberrance was much more common, with about 50% of B-precursor ALL cases and most cases of T-ALL deviating from normal B-lineage or T-lineage marrow cells [81]. The authors concluded from the high incidence of immunophenotypic heterogeneity that aberrant differentiation is frequently involved in leukemogenesis [81].

Recent studies using three- or four-color immunophenotypic analysis have confirmed a high incidence of immunologic aberrance in ALL. A recent study of 264 consecutive de novo precursor-B-ALL cases, using standardized flow cytometric protocols and reagents from laboratories in six countries (European BIOMED-1 Concerted Action), revealed immunophenotypic aberrance in nearly all cases when four or five triple monoclonal antibody combinations were used [82]. The incidence of cross-lineage antigen expression was 45%, mostly due to aberrant coexpression of CD13 or CD33. A 91% incidence of immunophenotypic aberrance was found in T-ALL. In a similar recent study, phenotypic aberrations were found in 88% of cases of B-lineage ALL and all T-ALL patients. Two or more

aberrancies were found in 75% of patients. Overall, there was a 50% incidence of lineage infidelity, a 68% of asynchronous antigen expression, a 4% incidence of antigen overexpression, and a 28% incidence of ectopic phenotype [78].

Explanations for the origin of unexpected phenotypes vary [83,84]. Some may represent malignant transformation of early “normal” multipotent hematopoietic cell populations or rare subpopulations of “normal” progenitor cells which coexpress antigens of different lineages (ie, “mixed lineage”, “multiple lineage”, etc). Others may represent aberrant stemlines that reflect abnormal patterns of differentiation (ie, “lineage infidelity”, “lineage promiscuity”, etc). The terms “asynchronous antigen expression”, “biphenotypic leukemia”, “metachronous leukemia”, “leukemias of indeterminate lineage”, “multiple lineage reactivity”, “immunophenotypic aberrance”, “marker discrepancy”, “ectopic antigens”, etc also have been used by different investigators [37,81,85–88].

Regardless of their origin and nomenclature, these cell populations often pose difficulties in the interpretation of flow cytometric data. Fortunately, certain patterns of aberrant antigen expression have been described, and common principles of interpretation can be applied. The prognostic significance of unusual antigen expression in acute leukemia is controversial, partially because of the lack of large-scale studies. Definitions of various terms used for aberrant antigen expression are presented in Table 3.

Aberrant antigen expression in B-ALL

The co-expression of myeloid cell surface antigens found in approximately 30–45% of children and adults with B-lineage ALL, and these neoplasms are termed myeloid surface antigen-positive (My+) ALL [82,89,90]. The expression of T-lineage antigens is less common in B-ALL. CD33 was the most common form of aberrant antigen expression in My+-ALL, followed by CD13, CD11b, and CD7 [228]. A correlation of My+-ALL with CD10 expression and L2 morphology has been found, while mature B-cell ALL cases are usually negative for myeloid antigen expression [88,90,91]. Myeloid-associated antigens are most common in childhood precursor B-cell ALL cases, and have a lower incidence in adult B-ALL patients. In precursor B-ALL, CD33 was identified by Bradstock et al as the most common form of aberrant antigen expression (11%), followed by CD13 (5%), CD11b (4%), and CD7 (2%) [92,93]. The t(9;22) translocation is the most common cytogenetic finding in My+-ALL, followed by 7q-, abnormalities of 11q with or without a translocation, 20q-, and -5 [79,90,94,95]. Immunoglobulin heavy chain gene rearrangements are also common in cases of My+-ALL with T-cell-phenotypes [89].

Subgroups of My+-ALL patients have been found which may have unique clinical and biologic features. For example, in one study of children with B-ALL, CD33 expression was identified in 15% at initial diagnosis and in 37% at relapse. CD33+ patients were statistically older than those without CD33 expression, and tended to have a poor outcome, although CD33 expression was not an independent predictor of survival in multivariate analysis. CD33 expression was

Table 3
Terminology for aberrant antigen expression^a

Term	Definition
Asynchronous antigen expression	Coexpression expression of early and late differentiation antigens in abnormal combinations (usually B-cells)
Intralineage aberrancy	
Biclonal acute leukemia	Leukemias arising from separate transformation events in distinct stem cells (not true hybrid)
Bilineal acute leukemia	Simultaneous distinct populations of leukemia cells of more than one lineage (usually one myeloid and one lymphoid)
Bilineage acute leukemia	
Biphenotypic acute leukemia	Leukemia with lineage marked promiscuity at single cell level (single clone expressing both myeloid and lymphoid antigens)
Synchronous mixed-lineage acute leukemia	
Bilineal synchronous acute leukemia	Simultaneous distinct populations of leukemic cells of more than one lineage
Bilineal metachronous ("lineage switch")	Development of a new leukemia with a different phenotype in a patient (excludes therapy-induced second leukemia) (usually ALL followed by AML)
Metachronous mixed lineage	
Hybrid acute leukemia	Leukemia with co-expressing lymphoid and myeloid features (includes biphenotypic and bilineal leukemias).
"Mixed" lineage acute leukemia	Balanced co-expression of different lineage-specific antigens. Usually result from transformation of a pluripotent cell.
Leukemia-associated phenotype	
Lineage infidelity	Acute leukemias with single aberrant ("cross-lineage") antigen, usually a poorly specific antigen, includes ALLs with myeloid-associated antigens and AMLs with lymphoid-associated antigens
Ectopic phenotype	
"Minimally differentiated" acute leukemia	Leukemias arising from very early myeloid or lymphoid cells
Mixed lineage acute leukemia	

^a Modified and updated from [85] and [1,228].

associated with coexpression of T-cell antigens, including CD2, CD4 and CD7. It was proposed that CD33+ B-precursor ALL is derived from undifferentiated hematopoietic cells minimally committed to B-cell lineage and that the more frequent occurrence of CD33 expression at relapse may reflect clonal evolution or the expansion of a CD33+ minor clone [96]. Myeloperoxidase positivity was demonstrated in the bone marrow biopsy material of patients with precursor B-ALL, utilizing both immunoperoxidase staining and Western blot analysis [97]. These neoplasms frequently showed CD13 or CD15 coexpression and evidence of t(9;22) by karyotype or polymerase chain reaction analysis.

Aberrant CD2 expression has been reported in a small proportion (<5%) of patients with precursor B-cell ALL [98,99,100]. Patients with this entity usually have a good to excellent prognosis. A putative CD2⁺CD19⁺ biphenotypic normal counterpart has been identified in fetal liver and bone marrow [100,101].

Hurwitz et al [229] recently performed detailed immunophenotypic analysis in 113 cases of B-lineage ALL, and reported the existence of sixteen different complete immunophenotypes that did not correspond to normal B-cell developmental stages in the bone marrow. Overall, 78% of the cases showed “asynchronous” combinations of B-cell associated antigens. The authors postulated that the majority of B-lineage leukemias exhibit “developmental asynchrony” as compared to normal B-cells.

Lineage switch in acute leukemia is a rare phenomenon in which the leukemic cell lineage changes from myeloid to lymphoid, or from lymphoid to myeloid. Among 89 cases of childhood leukemia in relapse, Stass [102] identified six (7%) cases of lineage switch documented by cytogenetic and immunophenotypic data. In five cases the lineage switch was from ALL to ANLL, while one patient converted from ANLL to ALL. Patients treated with agents specific for the leukemia phenotype at relapse readily achieved second remission, while those treated for the original phenotype failed to achieve remission. Postulated mechanisms for lineage switch include: (1) eradication of the dominant clone present at diagnosis, with expansion of a secondary clone with a different phenotype, and (2) drug-induced changes in the original clone with amplification or suppression of differentiation programs permitting phenotypic shift [102].

Aberrant antigen expression in T-ALL

A high incidence of intralineage antigen variation has been found in T-ALL. For example, using triple antigen staining with TdT/CD7/cyCD3 and four other antibody combinations, the investigators in the European BIOMED-1 Concerted Action found at least one aberrant pattern 91% of T-ALL cases [103]. In most cases, the aberrant neoplastic cells were found in areas of the flow cytometric histograms normally devoid of cells, so that identification of disease persistence and relapse could be readily identified. Overall, chromosome translocations involving regions of T-cell receptor genes are present in about 24% of T-cell ALL cases [90]. The existence of a common myeloid/T-lymphoid progenitor cell is suggested by similar patterns of TCR delta rearrangements in cases of myeloid-positive T-ALL and lymphoid-positive AML [104]. Interlineage antigen variation is less common in T-ALL than in B-ALL, but is found in about 10% of T-ALL cases. Among the reported cases of this phenomenon, HLA-DR is the usual “promiscuous” antigen, but some cases of myeloid-positive (My+) T-ALL have been reported. For example, Nakase et al reported a case of CD7+CD3–CD4+CD8–CD13+CD34+ ALL (FAB-L2) with germline immunoglobulin heavy chain and T-cell receptor beta, gamma and delta chain genes. Karyotypic analysis revealed complex abnormalities involving chromosomes 5 and 7 [105].

Hematogones

Hematogones are B-lineage lymphoid precursor cell populations in the bone marrow that may simulate acute lymphoblastic leukemia (ALL) or lymphoma.

These precursor lymphoid cells are homogenous with condensed chromatin and scant cytoplasm and may account for greater than 50% of bone marrow cells. They are found in large numbers in normal infants and occasionally in adults in a variety of hematologic and nonhematologic disorders [106,107].

Hematogones may express an antigen profile similar to that of neoplastic lymphoblasts (eg, TdT, CD34, CD10, and various pan B-cell antigens); however, Rimsza et al reported that hematogone-rich lymphoid proliferations exhibit a spectrum of B-lymphoid differentiation antigen expression with predominance of intermediate differentiated cells (CD19+, CD10+) and mature B-lineage cells (surface immunoglobulin), heterogeneity of adhesion molecule expression (CD44, CD54), and nonclustered bone marrow architectural distribution [107,108]. The authors found that the expression of CD34, terminal deoxynucleotidyl transferase (TdT)-positive cells as the smallest subset in a non-clustered pattern [108]. Hematogones, therefore, differ from neoplastic lymphoblasts by displaying a maturational spectrum of antigen expression, whereas ALL displays an incomplete maturational spectrum and antigen aberrance. In addition, there is no evidence of clonality by either cytogenetic or immunogenetic analysis in hematogones and the DNA content is normal. Hence, the expression of any of the pan B-cell markers in isolation is not synonymous with new onset ALL or relapse. Muehleck et al noted that TdT-positive cells in the bone marrow of a patient with ALL in remission following chemotherapy or bone marrow transplantation do not necessarily denote relapse [109].

The distinction of leukemic blasts from hematogones can be difficult by morphologic examination alone. Even though hematogones express CD34 and TdT, albeit in small quantities, this is usually in a non-clustered fashion [108]. Clusters of more than five CD34 and/or TdT-positive cells may be used to differentiate hematogones from lymphoblasts [110]. However, Kallakury et al reported that flow cytometric monitoring of post-therapy bone marrow specimens from patients with precursor B-cell acute lymphoblastic leukemia, is inferior to polymerase chain reaction (PCR) and concluded that PCR for antigen receptor gene rearrangements is a valuable and specific tool, helpful in differentiating hematogones from minimal residual disease in patients with treated ALL whose bone marrow harbors increased precursor B-cells (hematogones) [111].

Bone marrow regeneration

Because of the toxicity of agents used in preparing the marrow for transplantation, transplanted marrow requires years to return to a healthy pretransplant state. The myeloablative and conditioning regimens that purge malignant progenitors from the marrow also purge and damage non-malignant hematopoietic and stromal progenitor cells resulting in a diminished capacity for transplanted and native stem cell renewal [112]. This deficit is not always apparent from examination of a post-transplant peripheral smear or marrow biopsy. A prolonged, severe deficiency of erythroid and megakaryocyte marrow progenitors may persist for many years post-transplant although peripheral blood cells and marrow

cellularity have reached pre-transplant levels. Colony forming units-fibroblasts (CFU-f) the precursor stromal compartment for cells of the osteogenic lineage are critical to hematopoietic cell survival, proliferation and differentiation. CFU-f reconstitution may take as long as 12 years to reach pre-transplant numbers and is solely of host origin [113].

The immediate post-transplant period is usually followed by 2 to 3 weeks of marked marrow aplasia. By about 14 days post-transplant, aggregates of hematopoietic precursors may be identified within the marrow. These are single cell-type clusters of erythroid, myeloid, and undifferentiated colonies [114]. Hematopoiesis post-transplant is usually polyclonal but may be monoclonal [115]. The kinetics of engraftment in part depends the source of donor cells (peripheral blood stem cells [PBSC], cord blood, bone marrow), the dose of infused CD34+ cells, the administration or the withholding of exogenous growth factors, and HLA cross-matching [116]. The rate of marrow recovery is affected by the homing efficiency and clonogenic potential of transplanted cells as well as whether infused cells were expanded in vitro prior to infusion [117]. In vitro expanded cells appear to have a reduced capacity to locate the marrow as compared to fresh progenitors, which may compromise their biologic and clinical utility [118]. G-CSF-mobilized PBSC are coming into wider use as an alternative to autologous bone marrow transplantation. Trials with PBSC demonstrate earlier engraftment of neutrophils, platelets, and red cells when using PBSC without an increased risk of developing acute graft-versus-host disease (GVHD), though the risk of chronic GVHD however is somewhat increased [119]. Mouse studies suggest that PBSC transplantation brings about rapid hematopoietic recovery with partly impaired B-cell immune reconstitution in the first month, and low helper T-cell numbers for up to 12 months [120].

Evidence of marrow engraftment may be found first in the peripheral blood and is heralded by the return of high fluorescence reticulocytes (HFR). HFR are quickly reduced by ablative regimens, yet quickly recover after autologous or allogeneic bone marrow transplantation before the emergence of neutrophils. Automated flow cytometric reticulocyte counters are properly used to monitor rising HFR numbers since manual estimation of their presence and numbers is unreliable. Failure of mean reticulocyte volume (MRV) and reticulocyte mean fluorescence intensity (MFI) values for HFR to increase early on post-transplant may indicate primary graft failure and identify a group of patients who might benefit from early rescue [121]. Restoration of granulopoiesis occurs more or less simultaneously with erythropoiesis, reaching full marrow cellularity by 30 days post-transplant. CD8 T-cells reach pre-transplant levels early and exceed normal levels during the first several months, whereas CD4 T-cells require six to eight months to recover. NK cell activity recovers quickly. Non-myeloablative treatment before allogeneic transplantation permits near normal T-cell-dependent non-MHC restricted mitogenic responses even in the early period after transplant among patients with hematologic malignancies, while myeloablative conditioning results in decreased T-cell mitogenic capacity and slower immune recovery [122]. Non-myeloablative conditioning also may permit faster development of

immune response to residual host malignant cells and non-malignant abnormal hematopoietic cells. B-cell numbers reach pre-transplant levels by the second month and exceed normal after six to seven months.

Confirmation of engraftment may be achieved by blood cell isoenzyme studies, determination of red cell antigens, chromosome analysis, and assessment of immunoglobulin allotypes and DNA restriction fragment length polymorphisms. Bone marrow transplant donor-host chimerism may be assessed by PCR or by analysis of fluorescent labeled variable number of tandem repeats (VNTR) which appears to offer improved turnaround time, requires less DNA, and provides superior resolution and sensitivity over the former method [123].

Minimal residual disease

Minimal residual disease (MRD) is the persistence of leukemic cells in the bone marrow of other tissues after remission induction that is below the limit of detection by conventional morphologic assessment. Presently, it is believed that these residual leukemic cells are the possible source of disease relapse in many patients who achieve “complete” morphologic remission from ALL and other forms of leukemia. Unfortunately, the clinically relevant level of sensitivity for MRD detection has not been established, nor has it been documented that additional therapy to eradicate very small numbers of residual cells improves survival for patients in clinical and morphologic remission.

Laboratory techniques for the detection of minimal residual disease must meet four criteria, which include sensitivity (detection limit of at least 10^{-3} cells), specificity (ability to differentiate normal and malignant cells), reproducibility, and applicability (easy standardization and rapid collection of results) [124,125]. Morphologic evaluation, with an overall detection limit of approximately 5%, is clearly not suitable for the detection of minimal residual disease [126]. However, immunophenotypic analysis, cytogenetics, fluorescence in situ hybridization (FISH), Southern blotting, polymerase chain reaction (PCR), and other techniques with detection limits of 10^{-2} to 10^{-4} cells have been applied, as well as the clonogenic assay, which has a detection limit of $\leq 10^{-4}$ [124]. The techniques and application of minimal residual disease have been recently reviewed by several authors [103,124,127–129].

Flow cytometric analysis is less sensitive than the polymerase chain technique for MRD, but it is simple and rapid to perform, provides quantitative data, and has adequate sensitivity in many leukemia cases. The detection of minimal residual disease in leukemia by flow cytometric analysis is based on the presence of aberrant immunophenotypic features that are not characteristic of normal cell populations in the specimen under study. For example, the discovery of CD10+, TdT+, or CD34+ cells in the cerebrospinal fluid is diagnostic of MRD, since immature leukocytes with these markers are not normally present in the CSF. The expression of TdT, cytoplasmic CD3, CD1a, or the dual phenotype CD4+/CD8+ by bone marrow cells is diagnostic of residual MRD in T-ALL, since cells with these phenotypes are normally confined to the thymus. The detection of B-ALL

MRD is more difficult, since small numbers of immature B-cells are normally present in the bone marrow. The majority of B-ALL cases have aberrant antigenic features, including cross-lineage antigen expression (ie, TdT, T-cell, or myeloid antigens), asynchronous antigen expression, or changes in the level of antigen expression (ie, “dropped” or overexpressed antigens).

Ryan and collaborators were among the first to use multiparametric immunophenotypic analysis for MRD detection in ALL. Utilizing FITC-labeled anti-CD10 and dual parametric flow cytometry, they were able to detect one CD10+ lymphoblast/100,000 peripheral blood mononuclear cells [130]. Subsequently, numerous investigators have confirmed the ability of two-color, three-color, and more recently, four-color immunophenotypic analysis to detect small numbers of phenotypically aberrant cells in ALL [131–140]. The search for new markers and techniques of immunophenotypic analysis for MRD is also underway by several investigators. For example, Weir and co-workers used two four-color combinations of antibodies (CD19-APC/CD45-perCP/CD20-PE/CD10-FITC and CD19-APC/CD45-perCP/CD9-PE/CD34-FITC) to identify reproducible patterns of antigen expression in normal bone marrow. When applied to specimens with precursor B-cell ALL, 81/82 cases (99%) showed cell populations outside of the normal geometrical regions consistent with MRD. Dilution experiments revealed a sensitivity of 10^{-4} [141]. Ito et al stressed the superiority of using CD45/SSC gating rather than FSC/SSC gating for MRD detection, and reported a sensitivity of 10^{-5} , which was 14-fold higher than the conventional procedure [80]. Chen and co-workers reviewed a variety of proteins that show significant overexpression in B-ALL and found MRD estimates with CD58 to correlate well with those of polymerase chain reaction amplification of immunoglobulin genes [142]. Boccuni and collaborators found dual staining for CD66c and CD10 to differentiate even low percentages of residual leukemic cells (CD10+/CD66c+) from normal regenerating early-B-cells (CD10+/CD66c-) in CD10+ early-B-ALL following remission induction [143].

The availability of sensitive techniques for MRD detection has generated large amounts of data regarding the prognostic significance of small clones of residual leukemic cells following remission induction. Most of these studies have demonstrated that MRD detection is a powerful technique for the prediction of disease progression and outcome. In one study, the presence of $\geq 0.01\%$ at each time point was associated with a higher relapse rate, with patients having a high level of MRD at the end of induction or at week 14 showing a particularly poor outcome. The predictive value of MRD remained even after adjusting for adverse presenting features [144,145]. Another group evaluated the prognostic significance of residual disease after induction therapy and immediately prior to bone marrow transplantation. There was a 3.28 fold risk of relapse after bone marrow transplantation in the presence of leukemic lymphoblasts by multidimensional flow cytometry, even when morphologic evaluation and cytogenetic analysis was negative [146]. A few studies have not confirmed the prognostic significance of posttherapy monitoring of bone marrow specimens from patients with precursor B-cell ALL. Kallakury found no evidence of monoclonally rearranged antigen

receptor genes in 11 of 14 patients suspicious for MRD by multiparametric flow cytometric analysis [111]. Furthermore, ten of the 11 patients remained in remission at 73 months, suggesting that the flow cytometric findings were of a false-positive nature [111].

Body fluids

Central nervous system (CNS) involvement by acute lymphocytic leukemia is a significant clinical problem in spite of recent improvement in treatment regimens. Meningeal disease at initial diagnosis is a risk factor for later CNS and bone marrow relapse, a poor response to therapy, and decreased survival. In addition, isolated CNS relapses decrease the chance for survival and require treatment that has adverse consequences. The identification of leukemic cells in the cerebrospinal fluid (CSF) is a major means of diagnosing meningeal involvement. Conventional cytopathologic examination of Papanicolaou-stained cytospin preparations is the standard technique for the detection of CSF blast cells, but has a significance incidence of false-negative results [147]. Consequently, there is a great interest in ancillary techniques, including flow cytometric immunophenotypic analysis and molecular diagnostic methods, for blast cell identification.

Immunophenotypic analysis of CSF specimens in leukemic patients has relied upon conventional methods of minimal disease detection in the bone marrow and other body tissues, including the recognition of maturation shifts, distribution shifts, and aberrant antigen expression [148–150]. In particular, monoclonal antibodies specific for TdT have been especially useful for the detection of CSF B-ALL lymphoblasts, while TdT and CD1a are helpful in cases of T-ALL [151]. These studies have also demonstrated an association of CSF involvement with the more developmentally mature antigenic phenotypes. In this regard, Donskoy and collaborators demonstrated that 88% of patients with CSF leukemic involvement demonstrated the CD22, CD23, cytoplasmic immunoglobulin heavy chain, and/or surface IgM markers [148]. A study using polymerase chain reaction (PCR) assays of the bone marrow in T-ALL patients with isolated CNS relapse demonstrated a high incidence of submicroscopic involvement, possibly indicating the bone marrow as the origin of disease resurgence that is first detected clinically in the CNS [152]. However, another study using multiparametric flow cytometry and blast colony assays did not confirm this association, suggesting that other mechanisms may be involved in the etiology of leukemic CNS involvement [153].

Prognostic significance of immunophenotypic analysis of all

Acute childhood leukemia is one of the few success stories in oncology, with a cure achieved in more than two-thirds of patients with conventional chemotherapy. Presently, permanent remission (“cure”) occurs in about 70% of children with ALL, and 30–40% of those with AML treated with modern chemotherapeutic regimens [83]. The improvement of treatment outcome in leukemia has

increasingly resulted from the identification of subgroups of acute leukemia with different clinical, immunologic, and prognostic features, and from the application of subgroup-specific therapy. Although the relationship between the immunophenotype and other cellular features is not completely understood at this time, surface antigen expression is a major POG (Pediatric Oncology Group) criteria for the assignment of individual patients to treatment groups in order to predict prognosis, assess the relative effectiveness of different treatment regimens, and design leukemia species-specific therapy [154,155]. The significance of the immunophenotype in adult leukemia is more controversial, and the “cure” rate is only about 33% [156,157].

Prognostic significance of immunophenotype in B-ALL

Age and leukocyte count at presentation are the most important prognostic factors for childhood acute leukemia [158]. Although immunophenotypic data is useful in the context of ALL diagnosis and classification, CD10 is presently the only leukocyte antigen with independent prognostic significance, or upon which specific therapeutic decision making is made. At present, the information for all diagnostic procedures is best used to determine the risk group of the patient, upon which therapeutic decisions are based. For example, experimental therapies may be justified for high-risk patients with ALL, while antimetabolite-based therapy might be employed to minimize long-term sequelae in children in favorable prognostic groups [158].

CD10 expression

The CD10 antigen commonly regarded as the most prognostically significant surface marker in childhood B-ALL, where it is used to divide B-precursor cell ALL into subgroups with excellent and good prognosis; however, not all studies have confirmed the prognostic significance of CD10 expression. In one large study, the overall incidence of CD10 expression was 94% in patients with B-lineage ALL and 40% in T-ALL. Although CD10 expression was associated with favorable presenting features, including age >1 year, lower leukocyte count ($< 50 \times 10^9/L$), or increased DNA content (leukemic cell DNA index > 1.16 , or hyperdiploidy > 50 chromosomes), it had no independent prognostic significance [159]. Children with weak CD10 have been reported to be older than those strongly expressing CD10, and to have a significantly higher incidence of splenomegaly [160]. Among adults, the incidence of CD10+(weak) ALL was significantly higher in female patients; however, in neither children nor adults was the heterogeneity of CD19 expression a statistically significant determinant of complete remission rate or disease free survival.

CD10+CD19+CD34+ immature B-progenitor immunophenotype has been studied as an independent prognostic factor in childhood ALL. For example, in a large cohort of 2028 children with ALL, the CD10+CD19+CD34+ was asso-

ciated with favorable characteristics and slightly better event-free survival for children with ALL, and a subset of infants with significantly better outcomes [161].

CD20 and CD45 expression

The prognostic significance of the intensity of surface membrane antigen expression has been extensively evaluated in the Pediatric Oncology Group (POG) Study. In large cohorts of children with newly diagnosed B-ALL, they found that both CD45 intensity > 75% and CD20 intensity > 25% correlated with significantly poorer outcome in children older than one year. In addition, CD45 intensity and CD20 intensity were independent of previously reported poor prognostic factors, including the National Cancer Institute (NCI) risk group, ploidy, trisomies of 4 and 10, and adverse translocations including t(1;19), t(9;22), and t(4;11) [162].

The lack of CD45 expression is a favorable prognostic factor in childhood B-ALL. This feature has been reported to be associated with an excellent response to multi-agent chemotherapy, lower leukocyte counts and serum lactic dehydrogenase levels, hyperdiploidy > 50, and a DNA index > 1.15 [163]. In this regard, Ludwig et al attributed the worse prognosis of the pre-pre-B or pre-B phenotype to the distinct biological (eg, chromosomal aberrations) and clinical features (eg, high tumor cell load) of these subgroups, and could not confirm the independent value of immunophenotyping in predicting outcome [157,164,165].

CD34 expression

The expression of the CD34 antigen has been identified as a favorable prognostic factor for both disease-free and overall survival in childhood ALL [166]. In addition, there was a significant association of CD34 expression with an absence of extramedullary involvement, lower LDH levels, lower WBC counts, and lower proliferative activity than CD34-ALL. Although Cascavilla et al [166] did not associate CD34 expression with clinical outcome in adult ALL, it was identified as an adverse prognostic factor in adult ALL in a study by Thomas and coworkers [167].

Aberrant antigen expression

B-ALL clones showing inter- or intralinear antigen expression are easy to detect by multiparametric flow cytometry, and their presence at diagnosis and at different treatment phases has been extensively evaluated as prognostic indicators. In general, the presence of interlineage aberrance in ALL appears to be an adverse prognostic indicator in comparison to patients with monophenotypic leukemias. In particular, during the past decade a number of investigators using different flow cytometric staining and analytic techniques have found that myeloid antigen expression in both childhood and adult ALL significantly correlates with short disease-free survival, a short duration of first remission, and a high relapse rate at all treatment phases [88,168–171]. In a recent study of adult patients with biphenotypic leukemia, it was concluded that their poor prognosis justified a

more aggressive treatment procedure, including high-dose AraC or the use of Pgp modulators for first-line therapy [172]. The association with myeloid antigen expression in ALL appears weaker, and there is no correlation with no significant differences observed between conventional and aberrant ALL in the distribution of sex, age, leukocyte count, hemoglobin concentration, platelet count, blast count, French-American-British (FAB) type, lymphadenopathy, or organomegaly [170]. In one recent study, the presence of >1% aberrant B-lineage cells in the bone marrow after induction therapy at two consecutive time-points predicted relapse in the majority of cases [173]. Hara et al identified CD33 expression as the strongest univariate predictor of event-free survival in precursor B-cell but did not have significance in multivariate analysis [96].

Recent advances in ALL therapy may have impacted the prognostic significance of immunophenotypic features. In one study of adult ALL, in patients treated with modified CALGB therapy incorporating central nervous system prophylaxis, cyclophosphamide and the early use of L- asparaginase into the backbone of daunorubicin, vincristine and prednisone, there were no significant differences in response rates, remission duration, or survival in those patients coexpressing myeloid antigens [174]. In T-ALL, the number of T-markers expressed was of prognostic significance, with patients expressing six or more markers having significantly longer disease-free survival and survival compared with patients expressing three or fewer markers. The Philadelphia chromosome was significantly associated with B-LIN ALL cases which coexpressed CD19, CD34, and CD10, while the majority of t(4;11) cases were CD19+, CD34+ but CD10– [174].

P-Glycoprotein expression

The resistance of tumor cells to one or more chemotherapeutic agents (chemoresistance) is one of the major obstacles to effective chemotherapy. Therefore, the discovery of the mechanism through which some tumor cells evade the effect of multiple chemotherapeutic agents (ie, “multidrug resistance”) generated great interest. Multidrug resistance was first described by Kessel and collaborators in 1998 [175]. Numerous reports of multidrug resistance were subsequently detailed in the literature, and laboratory studies suggested that the phenomenon occurred at the level of the plasma membrane. In 1972, studies by Dano indicated that the major mechanism of MDR was an energy-dependent physical extrusion of the drugs from the cell [176]. Subsequent studies by Ling and coworkers led to the discovery of a class of integral membranes (P-glycoproteins) as the site of the membrane efflux pump [177,178]. More recently, the amino acid sequence of P-glycoprotein has been elucidated, and the responsible gene (multidrug resistance gene, MDR1) has been identified and cloned [179]. Multidrug resistance was a common feature of a variety of human tumors, and explains many cases of incomplete or partial responses to chemotherapy, as well as the chemoresistance of recurrent tumors. In some tumors multidrug resistance is a *de novo* property of the neoplastic cells, while in other cases it is acquired after trials with one or more chemotherapeutic agents. MDR expression can be determined by four

techniques, including flow cytometry, immunocytochemistry, functional assays, and RT-PCR [180].

In contrast to many other human tumors, P-glycoprotein expression does not appear to be a significant prognostic indicator. Wuchter et al could not correlate the functional activity of P-glycoprotein with immunophenotypical subgroups, response to induction chemotherapy, relapse rate, or overall survival in adults with ALL, while Kanerva found no correlation with the white blood cell count, age, sex, cytogenetic characteristics, event-free survival, or overall survival in childhood ALL [181–183]. However, several different monoclonal antibodies are available for P-glycoprotein analysis by flow cytometry and/or immunocytochemistry, and multiple techniques have been described for measuring the functional activity of P-glycoprotein [184–188]. In view of discordant results among different analytic techniques and different laboratories, standardization of technology may be needed before definitive conclusions can be reached regarding the prognostic significance of MRD in ALL [189].

DNA index, apoptosis, and cell cycle-associated parameters

The incidence of atypical DNA content in leukemias and other hematologic neoplasms is lower than in neoplasms of the solid tissues. In ALL, aneuploid stemlines have been identified in approximately 25% to 50% of reported cases [190–195]. Many investigators have identified DNA aneuploidy as a favorable prognostic indicator in childhood ALL. In this regard, Look and collaborators found ALL patients with a DNA index >1.16 to have a significantly better outcome than analysis, the DNA index had prognostic significance independent of other commonly utilized parameters, including leukocyte count, race, and age. Furthermore, ALL cases with CD10 antigen expression showed a higher frequency of aneuploid stemlines than CALLA-negative ALL ($P < 0.003$) or ANLL ($P < 0.001$). These investigators found childhood ALL patients with near-tetraploid stem lines to form a distinct subgroup characterized by an older age, T-cell immunophenotype, cross-lineage expression of myeloid antigens, FAB-L2 morphology, and a high incidence of relapse or death from disease [196]. High values for DNA content, RNA content, and proliferative activity were reported in L3 leukemia/Burkitt's lymphoma [197].

Nearly all lymphoblasts have been reported to express bcl-2. There are no apparent fluctuations during the different phases of the cell cycle. High levels of bcl-2 are associated with CD34 expression in some studies, but bcl-2 expression was not shown a significant indicator of prognosis in ALL patients by several investigators [198,199].

Other immunophenotypic parameters

The CD95 (Fas/APO-1) antigen is a 40–50kDa cell surface glycoprotein and a member of the NGF receptor superfamily that is expressed on a variety of cell lines, including most leukocytes. Anti-CD95 antibodies induce apoptosis in

activated T- and B-cells, NK-cells and thymocytes through the Fas signaling mechanism [200–202]. The Fas system is an integral part of the normal cell death machinery, and aberrant CD95 expression may be involved in tumor growth. A benign autoimmune lymphoproliferation syndrome (ALPS) in humans has been associated with mutations of CD95 [203]. The CD45 antigen is expressed in T- and B-cell ALL, with a higher level of expression in T-ALL [204,205]. In pre-B ALL, CD95 antigen expression on blast cells was a favorable prognostic sign associated with CD10 antigen expression and an increased relapse-free and total survival [206].

The CD9 (BA-2) antigen is a 24–27kDa glycoprotein and a member of the tetraspanin (TM4) superfamily of membrane proteins [207,208]. The CD9 antigen has been implicated in cell regulation and aggregation in B-cells and platelets, and as a regulator of cell motility in a variety of cell lines. The lack of CD9 and CD20 expression (or only partial positivity of these antigens) was reported to be 88% sensitive and 71% specific for the t(12;21)(p13;q22) translocation and TEL-AML1 rearrangement, with a positive predictive value of 47% [209].

The adhesion markers include the beta 1-integrins (CD49c, CD49d, CD49e, CD49f), β 2-integrins (CD11a, CD11b, CD11c), CD44, and CD54. The prognostic significance of adhesion marker expression is the topic of several recent studies. Although Hara and collaborators found the frequencies of CD11a, CD49f, and CD44 expression to be significantly higher in CD34⁺ pre-B childhood ALL than in CD34[–] pre-B ALL, only CD54 expression had independent prognostic significance. The presence of CD54 on the cell surface was an adverse prognostic factor associated with a poor prognosis. The estimated 5-year event-free survival was 42.3% for CD54⁺ B-ALL compared with 70.3% for CD54[–] patients ($P < 0.05$) [210].

Prognostic significance of immunophenotype in T-ALL

In a study sponsored by the Pediatric Oncology Group (POG), recursive partitioning analysis revealed a WBC level of $< 50 \times 10^3/\mu\text{L}$ as the most important single favorable prognostic factor in childhood ALL [211]. In patients with WBC levels of $< 50 \times 10^3/\mu\text{L}$, expression of CD5 was the most important predictor of event-free survival, whereas expression of the THY antigen was important at higher WBC levels. Pui and colleagues found CD10 expression to be an independent prognostic marker in T-ALL, with the lack of CD10 expression independently associated with an adverse prognosis ($P = 0.02$) [159]. In their study of 90 childhood T-ALL patients, there were no differences between CD10[–] and CD10⁺ cases in clinical features or karyotypic patterns, with the exception of a lower frequency of central nervous system leukemia and a higher frequency of 9p abnormalities in the former subgroup. CD7+CD4[–]CD8[–] acute leukemia is a distinct clinical subtype of T-ALL with an especially poor prognosis [158]. These leukemias predominately affect males (< 35 or > 65 years) who present with mediastinal and/or thymic masses, a high peripheral WBC count, and skin and CNS involvement [212].

The prognostic significance of myeloid antigen expression in ALL is controversial. Although it has been identified as an adverse prognostic factor in both childhood and adult ALL by most investigators, its independent prognostic significance is doubtful. Bradstock et al found that unusual antigen expression in precursor B-ALL was a good prognostic marker, while HLA-DR expression in T-ALL was associated with a poor disease outcome [92,93]. Wiersma and collaborators [171] found that myeloid-antigen expression significantly decreased the estimated disease-free survival, while Pui et al [213] found that myeloid antigen expression had no prognostic significance. In adult patients with ALL, Boldt et al found that the mortality rate was 85% greater in cases of myeloid positivity (MY+ ALL) in comparison to conventional ALL [91]. Sulak et al found that 7 out of 118 cases of adult acute leukemias that were biphenotypic for myeloid and B-lymphocytic surface markers behaved more aggressively than the others [88]. Boucheix and collaborators correlated CD10 coexpression in adult T-ALL with better disease-free survival, compared with the CD10-T-ALL [214].

The T-ALL phenotype CD1–CD2+CD4–CD7+CD10– has been associated with the t(1;14)(p32;q11), an alteration in the TAL1 gene (tald rearrangement), and very good to excellent prognosis [215]. A subgroup of CD3+4–8– T-ALL expressing the $\gamma\delta$ T-cell receptor has been identified. In contrast to the more common type of TCR $\alpha\beta$ T-ALL, patients with TCR $\gamma\delta$ leukemia present at a very young age with very high white blood cell counts, and no mediastinal enlargement [216].

In adult leukemia, older age, high WBC count at diagnosis, non-T-cell immunophenotype, Philadelphia chromosome (Ph)-positive karyotype, mediastinal mass, anemia, CNS involvement, and longer time to achieve remission have been identified as risk factors for systemic relapse [214]. Unfortunately, one or more of these features are present in the majority (60–70%) of patients with adult ALL, with a potential cure rate of 20% to 25%. In contrast, the cure rate is much higher (20–25%) in the minority without risk factors [217]. Short-term, dose-intensive therapy that alternates hyperfractionated doses of cyclophosphamide with high-dose cytarabine (ara-C) and methotrexate has been advocated for patients with mature B-ALL, while ara-C/cyclophosphamide combinations during maintenance therapy are currently favored for patients with T-cell.

Congenital, infant, and adult ALL are special subtypes that show different biologic behavior than classic childhood ALL. In infant ALL, Basso and collaborators correlated event-free survival with older age, relatively low WBC count ($<100,000/\text{mm}^3$), CD10 expression, and the lack of myeloid antigen expression [218,219]. In adult ALL, risk factors include older age, high presenting white blood cell count, non-T-cell immunophenotype, Ph-positive karyotype, and longer time to achieve remission [214]. In this disease, the expected cure rate is 20% to 25% for the high-risk group, versus 60% to 70% for patients without risk factors [214]. Modification of the dose-intensity of induction and consolidation-intensification therapy, incorporating new anti-ALL agents into the regimens, improving autologous BMT results (eg, purging, conditioning regimens), and appropriately using risk-oriented investigational strategies have been suggested as possible strategies to improve outcome in adult-ALL [220,221].

Spath-Schwalbe et al [222] studied ALL in patients >59 years of age. The following subtypes were identified: common ALL (14/26), T-ALL, six had null-ALL, three had myeloid antigen-positive ALL with both lymphoid and myeloid markers, and one had B-ALL. Although 43% of patients treated with chemotherapy achieved complete remission, the median survival was only 5 months. The major reason for the poor outcome was a high mortality during intensive induction therapy due to toxicity of treatment and a short remission duration [222]. Congenital leukemia is an aggressive disease that presents at birth or shortly afterward with a phenotype that can vary [223,224].

Summary and future prospects

ALL is a common hematologic malignancy characterized by the proliferation of immature lymphoid (blast) cells in the bone marrow. ALL leukemogenesis is not completely understood, but involves cellular and molecular dysregulation of the normal process of T- and B-cell growth and differentiation. ALL blast cells are phenotypically different from normal lymphoid precursors because of the disordered leukemogenesis, although ALL patients show great heterogeneity in the degree of variation. Until the 1980s, the diagnosis and classification of ALL relied upon morphology, cytochemistry, and karyotypic analysis in conjunction with clinical and routine laboratory data. More recently, the information obtained from multiparametric flow cytometric immunophenotypic analysis has become a critical part of ALL diagnosis. In addition, immunophenotypic analysis 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 the detection of minimal residual disease. Multiparametric, single cell immunophenotypic analysis by flow cytometry is performed with panels of fluorochrome-labeled monoclonal antibodies that recognize specific epitopes of different cell surface antigens. Single cell analysis of a statistically significant number of cells with the flow cytometer permits the recognition of maturation shifts, distribution shifts, or aberrant antigen expression that characterizes a leukemic cell population. Flow cytometric immunophenotypic analysis was initially performed by simultaneously labeling each cell with one or two different labeled monoclonal antibodies, but recent advances in flow cytometric instrumentation and computer technology now permit simultaneous labeling with three, four, or even five monoclonal antibodies per cell. As the progression of technology continues unabated, reagents and instrumentation under development today will undoubtedly result in further modifications of acute leukemia classification and diagnosis. One technology under evaluation at this time is laser-scanning cytometry, which permits slide-based, individual cell immunophenotypic analysis, morphologic evaluation, and morphometric studies. Laser scanning cytometry appears especially useful for the evaluation of fine needle aspirates and similar specimens where the small number of cells is a major disadvantage for flow cytometric analysis [225,226]. Eventually, conventional

flow or slide-based immunophenotypic analysis may be replaced by microarray analysis or similar techniques may permit the rapid, relatively inexpensive concurrent determination of more than 50 leukocyte differentiation antigens [227].

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