

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 Leucémies (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 mAbs 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 Intralineage aberrancy	Coexpression expression of early and late differentiation antigens in abnormal combinations (usually B-cells) Leukemias arising from separate transformation events in distinct stem cells (not true hybrid) Simultaneous distinct populations of leukemia cells of more than one lineage
Biclonal acute leukemia	(usually one myeloid and one lymphoid)
Bilineal acute leukemia	Leukemia with lineage marked promiscuity at single cell level (single clone expressing both myeloid and lymphoid antigens)
Bilineage acute leukemia	Simultaneous distinct populations of leukemic cells of more than one lineage
Biphenotypic acute leukemia	Development of a new leukemia with a different phenotype in a patient (excludes therapy-induced second leukemia) (usually ALL followed by AML)
Synchronous mixed-lineage acute leukemia	Leukemia with co-expressing lymphoid and myeloid features (includes biphenotypic and bilineal leukemias).
Bilineal synchronous acute leukemia	Balanced co-expression of different lineage-specific antigens. Usually result from transformation of a pluripotent cell.
Bilineal metachronous (“lineage switch”) Metachronous mixed lineage	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 Leukemias arising from very early myeloid or lymphoid cells
Hybrid acute leukemia “Mixed” lineage acute leukemia	Leukemia-associated phenotype Lineage infidelity Ectopic phenotype
“Minimally differentiated” acute leukemia	
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 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 of 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 chemotherapy 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 immunophenotypic 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 predominantly 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)(p22;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 disorder 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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