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Impact of immunophenotyping on management of acute leukemias

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ABSTRACT

Background and Objectives. **The diagnosis of acute leukemias (AL) requires a multiparametric approach in order to apply risk-adapted therapeutic protocols and appreciate the potential outcome of any given patient. Blast cells immunophenotyping is a key test in this issue, yet the information provided by immunophenotyping has become staggering, and it may be difficult to identify relevant characteristics clearly. This manuscript provides a critical review of the literature regarding the importance of immunophenotyping in acute leukemia diagnosis and management.**

Data sources and Methods. **The information given here is based on the experience of the authors, on their literature files and on additional material retrieved through articles and reviews covered by the Institute for Scientific Information (ISI) and the Medline® database. Studies with proper definition of the patients and sufficient information regarding follow-up were considered.**

Results. **Immunophenotyping allows an early confirmation of AL diagnosis and establishes lineage assignment. Adequate and comprehensive panels of monoclonal antibodies also allow detection of aberrant immunophenotypic profiles of prognostic value or of use in detecting minimal residual disease. A number of unusual immunophenotypic features are also associated with prognosis. The development of new antibodies, new insights in the functional properties of differentiation antigens, and the quantimetric approach of immunophenotyping will keep this field changing. Moreover, as therapeutic protocols evolve, some earlier results need to be reconsidered.**

Interpretation and Conclusions. **Immunophenotyping, together with cytologic, karyotypic and molecular approaches, retains a crucial place in the diagnosis and management of acute leukemias. It remains a rather specialized approach and should be interpreted in a multidisciplinary perspective, considering for each patient the idiosyncrasies possibly relevant to prognosis.**

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Since the development of monoclonal antibody technology in the late 1970's, immunophenotyping of neoplastic hematopoietic cells has proven to be of great clinical utility. Accordingly, the analysis of antigen expression has shown to be useful not only from a diagnostic point of view but also for prognostic evaluation and, more recently, for treatment monitoring of patients suffering from hematologic malignancies, including leukemias. It is well accepted that the final diagnosis of an acute leukemia should be based on a multifactorial approach in which clinico-biological, morphologic, cytochemical, conventional and molecular cytogenetics data, together with information on the immunophenotypic features of the leukemic cells are considered as a whole.

Here we make a critical review of the literature of the past 15 years, regarding the importance of immunophenotyping in the diagnosis and management of acute leukemia, as well as of the prognostic value of non-lineage markers' expression on blast cells.

The information given is based on the experience of the authors, on their literature files and on additional material retrieved through articles and reviews covered by the *Institute for Scientific Information* (ISI) and the Medline data base of the *National Library of Medicine PubMed*. Indexing terms such as acute leukemia or leukemia, immunophenotype (and truncatures), marker(s), survival and prognosis were used, and more specific searches were performed using the name(s) of the markers reported. Studies with proper definition of the patients and sufficient information regarding follow-up were considered.

The diagnosis of leukemias

Acute leukemias (AL), by definition, develop abruptly and require urgent management. The first diagnostic step is the enumeration of blast cells and

examination of their cytologic features.[1] Application of the French-American-British (FAB) classification at this stage gives a provisional diagnosis within a few hours of sampling. Identification of the cytochemical properties of blast cells has long been the only complementary information available. Detection of the myeloperoxidase enzyme still remains an important discriminative feature between acute lymphoblastic leukemias (ALL) and non-lymphoblastic AL (ANLL) also referred to as acute myeloblastic leukemias (AML).[2] Identification of the presence of functional non-specific esterase also provides useful information.[3]

In cases in which cell proliferation is successful, karyotypic analysis provides important additional data by showing specific chromosomal anomalies, some of them allowing diagnosis independently of other criteria.[2] In some instances, when metaphases cannot be obtained, molecular probes have become very useful in the determination of specific chromosomal aberrations.[4] However, it should be noted that the two latter approaches are not routinely available in a relatively large number of laboratories, and that they may fail to detect rapidly chromosomal or gene abnormalities in a substantial proportion of cases.

Since the early 1980s, with the exponential development of monoclonal antibodies, an increasingly complicated exploration of leukocyte immunophenotypes has become possible.[5] This has demonstrated the extraordinary variety of acute leukemias, and certainly provided confusion for some clinicians. Two types of attitudes can be observed in the literature regarding leukemia immunophenotype. The first attitude is that proper immunophenotyping is taken as granted. Acute leukemias are defined as of B or T lineage for ALL or as AML. Therapeutic stratification proceeds from this point and then disregards the immunophenotypic features of the blasts. The other attitude is to consider that due to the diversity of immunophenotypes possibly they have no impact on the disease outcome. This position is indeed somewhat difficult to counter, because few therapeutic trials are truly comparable on immunophenotypic grounds, protocol-effects are difficult to differentiate from relevant prognostic factors, and large studies often do not consider or allow for extensive immunophenotypic investigations.

Practically, and in spite of the fact that controversial attitudes can still be found in the literature, it is at present widely accepted that immunophenotyping provides a rapid and clinically useful tool to characterize neoplastic cells in AL patients.[6,7]

It should, however, be emphasized that some degree of variability exists in the quality and type of reagents used and methods employed in laboratories over the world. The criteria used for the interpretation of the results of immunophenotyping, together with the important degree of immunophenotypic heterogeneity of AL may lead to contrasting results in different studies and blur the value of immmunophenotypic analysis in the management of AL.

To some extent, the advances in molecular biology and molecular cytogenetics, which are the hallmark of the 1990s, have been taken into account better, although diversity in this field is also quite staggering.[4] The identification of unique chromosomal abnormalities has allowed identification of subgroups of patients with distinct outcomes, this concept being perhaps easier to deal with than complex immunophenotypes. Moreover, there seems to be some correlation, albeit not complete, between certain chromosomal abnormalities and FAB categories, and even immunophenotypic features,[8] therefore limiting the diversity and perhaps adding weight to prognostic features in such a context.

This does not mean that immmunophenotyping should be confined to confirmation of cell lineage - which in itself is no simple feat and requires the respect of some rules as detailed below – and/or that no more attempts should be made to evaluate the prognostic value of specific immunophenotypes. There are at least four reasons supporting the notion that immunophenotypic approaches should be pursued. The first one is that these investigations are feasible in all newly diagnosed patients. They do not require that cells grow, that DNA is well preserved or that probes hybridize properly. Second, the technology, if tricky, is rather sound and both reagents and machines have become highly reliable. Third, among patients with identical (or normal) karyotypes or genetic aberrations, the evolution of the disease is frequently heterogeneous and not predictable pointing to the need for additional information, some perhaps depending on the immunophenotype. An example of this is the similar clinical and laboratory features and poor prognosis of B-I ALL with or without t(4;11) translocation,[9] or the heterogeneity observed among AML cases with the t(15;17) translocation after treatment including all-trans retinoic acid (ATRA).[10] Finally, as will be exemplified below, in some instances, certain immunophenotypic profiles may raise the suspicion of chromosomal aberrations, and immunophenotypic data can be used to decide whether sophisticated molecular investigations should be undertaken.

Immunophenotypes of leukemias

Acute leukemias are characterized by an accumulation of hematopoietic cells blocked in the earliest stages of maturation, usually present in minimal numbers in normal bone marrow. Yet, the first stratification step in most therapeutic protocols is the diagnosis of either ALL or AML, and in the former group, the assessment of the B- or T-lineage of the leukemic cells. Proper lineage assignment is therefore of utmost importance, and it is currently agreed that demonstration of the intracytoplasmic expression of MPO, CD3 or CD79a allows the cells' lineage to be approached confidently, provided this expression is

confirmed by the demonstration of the surface expression of lineage-associated antigens. Although no real consensus exists, the current agreement is that the expression of close to 30 markers should be investigated in order to identify fully and properly, not only B and T lineage ALLs and AMLs (first diagnosis step), but also variant or biphenotypic acute leukemias in which markers of more than one lineage are coexpressed (full diagnosis step). Such a panel, and its use for the sub-classification of acute leukemias has been published by EGIL[6] as summarized in Table 1, and similar panels have been proposed by other groups.[7] Although only a few studies have reported on the degree of inter-laboratory concordance,[11] in most cases there is no ambiguity in the immunophenotype, with more than 60% of blast cells presenting with a characteristic immunophenotype allowing lineage assignment and full identification of the blasts idiosyncrasies. Yet, a few caveats should still be mentioned.

First, it is extremely important to perform intracytoplasmic labeling[10] as the most specific markers are expressed early and/or only in the cytoplasm. These are cyCD79 (usually cyCD79α), cyCD3 and myeloperoxidase (MPO), which are highly specific for the B-, T- and myeloid lineages respectively, and the intracytoplasmic expression of immunoglobulin mu chains, defining the B-III subset, a more mature stage of B-cell differentiation than "common" ALL or B-II.[6] Although cyCD79a appears to be the earliest B-lineage associated marker, cyCD22 has also been proposed for B-lineage assignment.[13] Analysis of CD13 or CD33 intracytoplasmic expression may also be helpful in the definition of poorly differentiated AML.[14] The detection of intracytoplasmic molecules is also more sensitive for the identification of megakaryocytic markers such as CD41, CD42 or CD61.[6] The techniques for intracytoplasmic labeling differ from those used for membrane staining, and require more rigorous controls.[15] Numerous types of membrane permeabilization have been described and are commercially available, which may yield significantly different data.[16] This is the reason why some laboratories are reluctant to set them up, in spite of their highly informative significance.[7] The lack of information on the expression of intracytoplasmic mu chains in many literature reports also hampers the interest of these studies and impairs proper meta-analyses.

Second, the increased sophistication of flow cytometry techniques has led to the development of multicolour labeling, which helps to establish a diagnosis even faster, but again should be used with discrimination and controlled technology.[7,17] An unexpected consequence of the use of phycoerythrin labeling, which yields brighter staining than fluorescein isothiocyanate, has been to increase dramatically the incidence of myeloid variants in ALL, i.e. cases with the aberrant expression of one or two myeloid antigens.[18]

Thirdly, an upcoming feature of immunophenotyping methods that might modify the prognostic significance of expression of several markers is the quantimetric assessment of the expression of differentiation antigens, providing an indication of their density on blast cells. Again, the methodology is slightly different from that used for routine membrane labeling. Antibodies must be used in saturating conditions, preferably in single-color techniques to avoid the use of fluorescence compensation, and calibrators must be used to express data uniformly.[19]

Table 1. Immunophenotypic markers allowing for lineage assignment and ALL sub-classification (adapted from ref. #6).

	Cytoplasmic	*Positive markers*	*Mandatory negative*
T-lineage			
T-I	CD3	CD7	All other T-lineage markers
T-II	CD3	CD7, CD2, CD5	CD1, CD3
T-III	CD3	CD1a	CD3
T-IV	CD3	CD3	CD1
B-lineage			
B-I	CD79a	CD19, CD22	CD10, cμ, sIg
B-II	CD79a	CD19, CD22, CD10	cμ, sIg
B-III	CD79a	CD19, CD22, cμ	sIg
B-IV	CD79a	CD19, CD22, sIg	
Myeloid lineage	MPO	CD13, CD33, CD117, CD65s, CD64, CD14, CD15	
Megakaryocytic lineage		CD61, CD41, CD42	
Erythroid lineage		CD36, glycophorin A	
Useful non-lineage markers		CD9, DR, CD38, TdT	

Stratification

Prognostic factors for the outcome of ALL and AML first take into account the age of the patients and their leukocyte count.[20] Infants, children, young adults and elderly patients are stratified into different categories, and protocols are first adapted to this feature.[21,22]

The second stratification after age is the blast cell lineage, i.e. confirmation of the leukemia being lymphoblastic or myeloblastic.[20] Cytologic features are useful as a first step, and often allow an initial classification according to the FAB recommendations. Immunophenotyping is however mandatory to define the lymphoid lineage involved and, in cases with undifferentiated blasts, to determine the myeloid subtype. A proper immunophenotyping strategy nowadays allows the diagnosis and classification of AL in over 99% of the cases. Acute undifferentiated leukemia (AUL), characterized by the absence of lineage-associated markers on blast cells, has thus become an extremely rare disease,[23] and multicenter studies are necessary to determine whether AUL truly represents a clinical entity.

Acute lymphoblastic leukemias (ALL)

Among ALL, B- and T-lineage cases also differ by their clinical and laboratory features, T-ALL usually being associated with high WBC counts, organomegaly and mediastinal mass.[24] Childhood T-lineage ALL has a poorer prognosis than B-lineage ALL, and requires more intensive therapeutic regimens.[25,26] Recent reports indicate a significant improvement in event-free survival (EFS), up to 70% at 7 years.[24] In adults, T-lineage ALL is currently claimed to be have a better prognosis than B-lineage ALL.[27]

Further stratification is provided by the immunophenotypic subclassification of both B- and T- lineage ALL, allowing the implementation of specifically designed protocols which allow for high recovery rates. This attitude has radically changed the prognosis of Burkitt-like ALL or B-IV over the past decade from a lethal disease to a curable one.[28,29] The most undifferentiated B-ALL, or B-I, lacking the expression of CD10 and associated in close to 25% of cases with the t(4;11) translocation, has been recognized to have a poorer prognosis in many studies.[9,30,31] The common B-II ALL, representing over 60% of childhood ALL[1] is considered to have a good prognosis with about 85% of children achieving a long-term remission,[32] while it is associated with a worse outcome in adults.[27] This large group is however heterogeneous in term of response to therapy, and should be further subdivided immunophenotypically as well as according to cytogenetic and molecular markers. Less information is available on the outcome of B-III ALL, characterized by the presence of intracytoplasmic mu chains, but some studies identified this immunophenotype as being associated with a poor prognosis.[25,33] This highlights the importance of the determination of intracytoplasmic μ chains to discriminate between B-II and B-III ALL.

Among T-lineage ALL, a worse prognosis is again associated with the most immature stage of pro-T ALL or T-I, both in adults and children.[24-26]

Acute myeloblastic leukemias (AML)

AML is a rare disease in childhood (about 15% of childhood AL), with a poor outcome recently improved with the use of intensive chemotherapy and bone marrow transplantation to about 50% survival at 5 years.[34]

AML is much more frequent than ALL in adults, yet also of poor prognosis,[2,22] especially in elderly patients.[35] Morphologic criteria of the FAB classification allow the identification of blast cells of the various myeloid lineages. Immunophenotyping at diagnosis is especially precious for the identification of morphologically undifferentiated M0 AML cases, accurate diagnosis of the hypogranular M3 variants, and detection of blasts of megakaryocytic (M7) or erythroid (M6) lineages. This allows adequate identification of the nature of blast cells, permitting retrospective studies considering the outcome of well-defined patients, and, further, has therapeutic implications for M3 variants.[36]

Solary *et al.*,[37] through extensive immunophenotyping of 154 cases of adult AML, demonstrated the significant prognostic value of the $CD14^+/DR^-$ immunophenotype, independently associated with poor prognosis. The poor outcome of FAB M0-M3 AML patients with $CD14^+$ blasts has been recently confirmed.[38] Conversely, M3-AML, also designated acute promyelocytic leukemias are of good prognosis once recognized, since they can be cured with specific protocols with the addition of ATRA.[10,39]

Biphenotypic acute leukemia

The coexpression of differentiation antigens associated with two or more different lineages on blast cells is a relatively frequent feature in AL. When lineage assignment and, in ALL, immunophenotypic subclassification is clear, the presence of one or two aberrant markers from another lineage defines the leukemia as *variant ALL*.[6,40] In ALL, myeloid variants (My+ ALL) have been reported to be frequently associated with the t(4;11) translocation, and to have an unfavorable outcome in some series[41-43] but not in others.[44,45] However, the recent report of a significant correlation between myeloid antigens expression and TEL-AML1 fusion (resulting from the t(12;21) (p13;q22) translocation) in childhood ALL[46,47] suggests that immunophenotyping is a useful prescreening of molecular analyses, and that the outcome of My+ ALL should perhaps be reconsidered. The expression of markers considered to be lymphoid lineage-associated in AML is also relatively frequent, ranging between 10% and 25% of the cases.[18] Significantly poorer prognosis has been reported by some groups to be associated with CD7[18] or CD19[37] expression.

Truly biphenotypic AL are rare diseases, accounting for less than 5% of AL, when a proper definition is applied, i.e. high scores for more than one lineage using the EGIL proposal in which each informative marker has been given a weight expressed as "points". According to this, BAL is identified when more than two points are demonstrated in more than one lineage.[6,48,49] Little is yet known of the outcome of these patients, although preliminary data suggest a poor response to therapy, even assuming proper treatment is applied, which is difficult to devise for patients displaying features of both AML and ALL.[48]

Prognostic value of non-lineage associated markers

In addition to the stratification described above according to the blasts' lineage and differentiation stage, numerous studies have investigated the prognostic weight of individual markers, not associated to cell lineage and not included in the *minimal panel* considered for diagnosis and stratification (Table 2).

Many of these studies have to be considered in the therapeutic context of the time at which they were performed, since the improvement of therapeutic

protocols tends to modify the prognostic value of such features. Earlier studies should nevertheless be kept in mind, at a time when the trend is to apply lighter protocols to patients considered to have good clinical and/or cytogenetic prognostic features.[20]

Furthermore, as better understanding is gained of the functional role of cell proteins, additional studies are needed to evaluate the prognostic value of the presence or absence of newly defined molecules on blast cells.

Finally, an important parameter to consider when testing the significance of a given immunophenotype is the positivity threshold taken. In most studies this threshold is set around 10-30%,[6] yet the interpretation of cytograms may vary in determining the percentages of positive cells.[50] Other studies consider the expression of a given marker on blast cells as linear, and deal with all values observed.[51] The proposal by Paietta *et al.*[52] allows for a retrospective identification of prognostically significant expression levels. The recent development of cell labeling quantification has begun to raise interest as being yet another different approach to immunophenotypic features.

CD34

CD34 is a transmembrane glycoprotein, heavily glycosylated and particularly rich in O-linked carbohydrates and sialic acid, suggesting a mucin-like structure. It is expressed on early undifferentiated hematopoietic progenitor stem cells, and remains expressed on committed progenitors over several stages of myeloid and lymphoid maturation in leukemic cells.[53] The prognostic value of CD34 expression has been widely explored, and appears to depend on the type of leukemia examined and type of treatment applied.[54,55] CD34 expression, tested in multivariate analysis, was found to be an independent positive prognosis factor in childhood ALL,[56-58] whatever the lineage involved. In AML, CD34 is frequently expressed, except on M3 and M4 blasts.[37,55] The prognostic value of CD34 expression in AML is opposite to that reported in childhood ALL, and has been shown to be associated with a worse prognosis.[37,59,60]

A problematic issue in assessing the prognostic value of CD34 is the fact that this molecule displays different types of glycosylation, identified by three classes of monoclonal antibodies. Most of the studies reported above used only one monoclonal antibody, yet variations were reported, according to the FAB subtype of AML, in the expression of the various CD34 classes.[61,62]

CD45

CD45 is a pan-leukocyte antigen, displaying alternate splicing yielding 5 different types of surface molecules.[63] Antibodies directed to the framework structure of CD45 recognize all isoforms of the molecule. CD45 is usually expressed on all normal hematopoietic cells except mature erythroid cells. In a study of 258 consecutive children with ALL, Behm *et al.*[64] observed that the absence or low expression (<25% blasts) of CD45, in 32 patients, was associated with good prognostic features and a better outcome. Ratei *et al.*[65] also observed differences in the expression of CD45 on childhood ALL blast cells, but noted no relationship with the outcome in a homogeneous therapeutic protocol.

CD9

The tetraspan molecule CD9, identified on platelets, was originally described as a B-lineage associated antigen, useful for the classification of ALL. It was later demonstrated that CD9 had a much wider distribution, both on several types of tissues and on leukocytes.[66] The absence of CD9 expression in AML has been proposed as an immunophenotypic feature

Table 2. Association between non-lineage markers expression on blast cells and prognosis.

	ALL		*AML*	
Marker	*Univariate analysis*	*Multivariate analysis*	*Univariate analysis*	*Multivariate analysis*
CD34	Positive, children (57,58)	Positive, children (56)	Negative, adults (37,59,60)	Negative, adults (37,59)
CD45	Negative, children (64)			
CD9	Positive, children. (46)		Negative, adults (68)	Negative, adults (68)
CD2	Positive, children (69)	Positive, children (69)		
CD11b			Negative, adults (52)	Negative, adults (70)
CD44v6			Negative, adults (73)	
CD56			Positive, adults (75)	
			Negative, adults (74,75)	
CD58	Positive, adults (78)	Positive, adults (78)	Positive, adults (78)	Positive, adults (78)
CD54	Positive, children (79)			
CD95			Positive, adults (80,81)	
Pgp			Negative, adults (89,90)	Negative, adults (89)
LRP			Negative, children (92)	

suggestive of t(15;17) AML M3.[67] In line with these findings, early reports suggested a bad prognosis for CD9+ AML cases.[68] More recently, in childhood ALL, the absence or low expression of CD9 was reported to be highly predictive of a TEL-AML1 rearrangement[46] and therefore a good prognostic marker. According to this report, immunophenotypic prescreening could eliminate the need for molecular testing in patients with strong CD9 expression.

Adhesion molecules

The expression of adhesion molecules, potentially involved in cell-cell interactions, is often considered in oncology. These molecules could be involved in triggering cell death signals leading the blasts towards apoptosis, or favor cytotoxic cell adhesion.

In acute leukemias, several adhesion molecules have been investigated for their potential prognostic value as follows:

CD2, or LFA-2, also known as the sheep-rosette receptor because of its affinity for CD58 or LFA-3, is physically associated with protein tyrosine kinases. CD2 expression is one of the discriminative parameters between T-I and T-II in the EGIL classification of T-ALL.[6] Uckun *et al*.[69] have reported that patients with CD2+ ALL have a better outcome than CD2– cases, independently of other risk factors. In this study, none of the other T-lineage antigens tested appeared to correlate with the patients' outcome.

Among integrins, *CD11b* has been reported to be a prognostic factor, probably in relation to patients with AML with monocytic differentiation.[70] In a recent study[52] it was found to be expressed in 95 out of 382 newly diagnosed AML, without any clear relationship with monocytic differentiation. These cells had a high degree of immaturity, and the expression of CD11b was independently and significantly associated with poor response to therapy and poor prognosis.[52]

CD44, a lymphocyte homing receptor involved in numerous cell functions, is in fact a complex molecule, displaying several variant isoforms generated by alternative splicing.[71] These variants, noted CD44v, are not usually observed on normal peripheral blood cells.[72] The expression of CD44v6 on AML blast cells was demonstrated by Legras *et al.*[73] to be independently associated with a poor prognosis.

CD56 is a cell adhesion molecule involved in nerve growth, but also expressed on a subset of natural killer cells, some T-cells and myeloid cells, as well as early progenitors.[74] The gene encoding CD56 is located on chromosome 11, at the q23 locus, a frequent leukemia-associated breakpoint region. Vidriales *et al.*[75] have suggested that its expression might be associated with a good prognosis in adult AML. However, Thomas *et al*.[51] found no correlation between CD56 expression and leukemia outcome. A more recent study by Baer *et al.*,[76] focusing on the specific subset of AML with t(8;21)(q22;q22), indicated that patients with CD56+ blasts fared significantly less well than patients whose blasts did not express CD56. The expression of CD56 was also indicative of poor outcome in a small series of 23 CD56+ AML patients among a larger cohort of 114 patients.[74] Natural killer-AL (NK-AL), which also express CD56 and may be misdiagnosed as AML-M3, are important to recognize as they do not respond to therapeutic protocols with ATRA.[77]

CD58 expression was examined by Archimbaud *et al.*[74] on both adult ALL and AML, and found to correlate with a better outcome. In this series, CD58 expression was observed in about 45% of the cases in both types of AL and independently associated with longer survival. This study[78] also examined the expression of CD54, a molecule less often observed on AL cells, which had no bearing on the patients outcome. In a different study, Mielcarek *et al.*[79] observed a better outcome for children with CD54+ ALL blasts.

Apoptosis-related molecules

Resistance to spontaneous or drug-induced apoptosis, once suspected, became a very interesting topic to explore in order to explain the bad prognosis of some patients with AL. Because of the complexity and tight regulation of programmed cell death, it has been much more difficult than initially expected to demonstrate a relationship between the expression or absence of apoptosis regulation key molecules and outcome. In ALL, extensive studies in Germany demonstrated that CD95 expression on blast cells was not, as initially expected, an indicator of sensitivity to apoptosis.[80] The absence of a clear relationship between CD95 expression and induction of apoptosis was also demonstrated in a series of AML by Ijima *et al.*[81] yet these authors observed a significant correlation between CD95 expression and the response to induction chemotherapy. Stoetzer *et al*.[80] also demonstrated a correlation between the bcl-2/bax ratio in AML and response to treatment. Similarly, a better response to therapy was noted by the same authors when ICE (interleukin-1β converting enzyme) expression was high in AML.

Multi-drug resistance

Demonstration of the molecular mechanisms involved in drug resistance, and especially the development of monoclonal antibodies directed to the P-glycoprotein (Pgp), have raised high hopes for early detection of patients liable to resist chemotherapy. The presence of multi-drug resistance-associated molecules can be detected by functional assays measuring rhodamine 123 (Rh123) efflux in flow cytometry, through the demonstration of surface expression of the molecules using several monoclonal antibodies or by the determination of transcription products with molecular biology techniques.[82] Recent consensus recommendations have been published, emphasizing the requirement that, for the success of clinical trials, multiple techniques be employed to ensure accurate measurement of Pgp expression.[83,84]

Multi-drug resistance markers and activity are usually expressed at a higher level in AML cases.[82,85,86] Nussler *et al*.,[85] studying 166 AML patients treated with the AML-6 protocol, demonstrated that Pgp overexpression at primary diagnosis or relapse had an inverse influence on AML-6 treatment outcome. Zochbauer *et al*.[87] observed a significant difference in the outcome of AML patients based on a 5% cut-off of positively labeled cells for Pgp assessed with the C219 monoclonal antibody. Several other studies[88-91] have reported a significant correlation between low Pgp function or expression of LRP (lung-resistance protein) and good prognosis in AML patients treated with standard chemotherapy. The expression of LRP was also shown to correlate with poor prognosis in childhood AML, in a study in which no prognostic value was found for Pgp.[92]

Only a few studies on ALL have been reported. Wattel *et al.*[93] found no correlation between Pgp expression and response to treatment in adult ALL while Goasguen *et al.*[94] observed a significantly higher rate of first complete remission and a lower rate of relapse in MDR-negative ALL patients. In this study, survival rates for both children and adults were significantly higher in MDR-negative patients. Similarly, Volm *et al.*[95] observed longer relapse-free intervals in childhood ALL with LRP-negative blasts.

Quantimetry

The routine use of flow cytometry in laboratories involved in AL immunophenotyping has given rise in the past decade to a growing interest in using the indications of fluorescence intensity provided by this technique. There are technical pitfalls that should be taken into account, and analysis of the emerging literature on this topic is often made difficult by obvious methodologic flaws. Ideally, quantimetric approaches should be restricted to single-marker analysis, using monoclonal antibodies in saturating conditions.[96] Variations in the fluorescent signals obtained may be related to the brand of flow cytometer, instrument set-up, affinity and fluorochrome/protein (F/P) ratio of the monoclonal antibody used.[19] Within a laboratory, comparisons are often made using the mean fluorescence index (MFI) to describe the fluorescence intensity of a given marker. The MFI is calculated as the ratio of the sample mean channel/isotypic control mean channel. In order to make data more comparable between laboratories, calibrators should be used to express data, according to reproducible standard curves, in mean equivalents of soluble fluorescein (MESF).

Using the MFI, Lauria *et al.*[97] found a correlation between the level of bcl-2 expression in AML blast cells and poor outcome, a feature that correlated with CD34 expression. The MFI was also used by Taskov *et al.*[98] in an attempt at stratifying the B-II subtype of ALL. These authors observed a significant correlation between the duration of complete remission and the levels of CD98 expression. In a large study by the *Pediatric Oncology Group*, a worse outcome was reported in children with ALL when blast cells had higher levels of CD45 or CD20, expressed as MESF, providing new, independent risk factors.[99]

Monitoring of minimal residual disease

Another impact of immunophenotyping on AL patients' management and outcome is that it provides tools for the appreciation of minimal residual disease.[100] This cannot be achieved if only a few markers are investigated, i.e. in order to merely confirm a suspected lineage assignment. Identification of specific features of leukemic cells relies on extensive exploration of their characteristics, associating immunophenotypic and chromosomal investigations.[101-103] This approach allows the detection of imunophenotypic aberrance, useful for the detection of persisting blast cells after therapy, that may be appreciated in single point studies, or, better, during follow-up. The persistence or gradual increase in the number of residual leukemic cells significantly correlates with a higher incidence of relapse and a poor outcome, both in AML[104] and ALL.[101,105,106] Among the approaches proposed, San Miguel *et al.*[104] recommend a multiparametric flow-cytometric analysis involving a large panel of monoclonal antibodies. Applied to 53 AML patients, this strategy made it possible to demonstrate significant correlations between the patients' outcome and the number of residual cells detected by flow cytometry in bone marrow samples which were considered in morphologic remission. In ALL, immunophenotypic detection of residual blast cells appears to represent a powerful tool for the prediction of relapse both in children and adults, with a high sensitivity regarding the detection of low numbers of leukemic cells ($\leq 10^{-4}$).[105-107] By comparison, the combination of FISH and bromodeoxyuridine labeling is claimed to allow the detection of 3 leukemic cells in 10^5 normal cells[108] and the use of polymerase chain reaction as low as 1 malignant cell in 10^6 normal cells.[109]

Some limitations to this practice still remain, mainly related to technical questions such as the use of large panels and availability of experienced personnel. It is also mandatory that the diagnosis sample displays traceable immunophenotypic aberrants. Yet, the integration of several methods, including immunophenotyping, is beginning to be proposed, for instance with the new concept of FICTION or combination of immunophenotyping and FISH.[110]

As an alternative, it has recently been suggested that both in AML and in B-I ALL the detection, by flow cytometry of abnormalities in the differentiation pathway is associated with a higher incidence of relapse and poor outcome.[105,106] This approach is independent of the availability of a diagnostic sample and could be considered for the follow-up of patients with poor response to therapy.

Conclusions

The immunophenotype of acute leukemias is indeed a highly diverse feature of these diseases. A virtual consensus has nevertheless been attained as to the necessary panel allowing diagnosis and sub-classification, including the proper detection of biphenotypic AL. Immunophenotyping data, as for any other clinical or biological characteristics of AL, cannot be used alone, and must be considered together with all parameters of any given patient. As therapeutic protocols improve, two types of attempts should be made. First, to identify features of good prognosis allowing the amount of chemotherapy to be decreased and therefore minimizing long-term side effects of these drugs. At the other end of the scale, every effort should be made to try and understand why patients with apparently common forms of AL fail to respond to validated protocols. Proper and thorough immunophenotyping may help both aims, assuming that specialized clinicians and biologists keep working together on these issues. Patients' samples should therefore be used i) to provide, rapidly, the minimum information necessary for diagnosis, stratification and risk assessment and ii) to explore the potential value of new approaches enforcing the prognostic significance of AL-related immunophenotypic features.

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MCB collected the information and wrote the first draft. Each of the other authors edited this first manuscript and provided further references or relevant material. Answers to the reviewers' comments and revision of the manuscript again involved all authors.

Authorship is according to alphabetical order of EGIL members, yet most of the work was indeed carried out by the first author.

Disclosures

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