Background and Objectives. Flow cytometry is nowadays the preferred method for immunophenotypic identification, enumeration and characterization of blast cells at diagnosis. Despite widespread application of standardized protocols, inter-laboratory reproducibility has still not been achieved. The complexity of diagnosis and evaluation of minimal residual disease, in immunophenotyping acute leukemia, demands the use of a test that provides all the necessary information.

Data Sources and Methods. The information given here is derived from the experience of the authors and from literature files. The most relevant studies with adequate conclusions were considered. We report on the current status of multiparametric immunophenotyping using simultaneous three and four-color staining and the applications of this technique.

Results. Multiparametric immunophenotyping is a powerful method for achieving a clear discrimination between normal and pathologic cells. The specific identification of leukemic cells by immunologic gating forms the basis for immunophenotypic diagnosis, classification as well as prognostic evaluation of patients with acute leukemias. The performance of the procedure with regards to the panels of reagents and the analytic processes, is necessarily different in lymphoblastic and myeloblastic leukemias, since the diagnostic questions are different. Phenotypic information should be specifically provided for the blast cells and antigen expression should preferably be reported in quantitative units and CV. This would allow a standardized cross evaluation of immunophenotypic results between different investigators and laboratories.

Interpretation and Conclusions. Recent reports indicate that phenotypic aberrations reflect genetic abnormalities of leukemic cells and therefore their definition and identification is of clinical relevance not only for minimal residual disease monitoring but also for subclassifying acute myeloid and lymphocytic leukemias.

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Key words: acute leukemia, diagnosis, immunophenotyping, protocols

A n adequate diagnosis is the first goal towards tailoring correct treatment for patients with acute leukemias. For a long time morphologic and cytochemical evaluations have been considered the principal diagnostic criteria and the FAB classification has been generally accepted, despite difficulties remaining in achieving inter-laboratory reproducibility.1-8 Immuno-phenotyping is considered to be very useful for lineage assignment of immature and mature leukemic cells.9-13 The commercial availability of large panels of high quality reagents facilitates the applications of this analytic method in diagnosis, classification, prognostic evaluation, and minimal residual disease evaluation.10-32 At present flow cytometric analysis is the preferred method for immunophenotyping while the role of microscopic evaluation of antibody-stained cells is reserved for exceptional situations. Flow cytometry allows the analysis of a large number of cells (usually between ten thousand cells per sample, and more than one million in minimal residual disease studies) providing high statistical accuracy. The simultaneous analysis of several different parameters (typically 5 or 6: two related to physical properties of the cells and 3 or 4 to the immunophenotype) contributes to increasing both the specificity and sensitivity of the test. Furthermore, the results can be stored in list-mode files and interpreted by other observers to facilitate objective interpretation. At present the two major sources of variability related to flow cytometric
immunophenotyping of leukemic samples are: the methods used for antigen expression assessment and the criteria employed for the interpretation of the results.

The first goal of immunophenotyping is the identification of pathologic cells; the second aim is phenotypic characterization.

Test principle

Immunophenotyping is based on the identification of specific patterns of both surface and intracellular antigen expression in unique populations of cells.11-13,18,22 Accordingly the phenotypic expression patterns of normal cells present in a sample analyzed for diagnostic purposes must have been established in advance.

This principle should be applied even when normal patterns are dynamic, such as in bone marrow, where the normal regenerating cells are present at various stages of maturation.33

Classically, leukemic blast cells have been considered to represent the neoplastic counterpart of normal immature cells blocked at a specific differentiation stage. At present this opinion needs to be reconsidered. The multiparametric, quantitative approaches have shown that expression in leukemic blasts is frequently aberrant in terms of both the presence of cross-lineage antigens (i.e. myeloid antigens in lymphoid cells and vice versa) and the presence of abnormal expression of normal antigens. This anomalous antigen expression is not directly related to an abnormal differentiation pattern but instead seems to reflect an underlying abnormal genetic pattern of these leukemic cells.13,22,25,32-40 Such phenotypic aberrations are essential for the identification of pathologic cells among normal cells.12,13 Initially light scattering properties of the cells were used for the specific identification of abnormal cells, but it is now well-established that this is not sufficient (Figure 1A).41

When associated with other immunologic markers, light scattering parameters are quite helpful in obtaining specific and sensitive identification of blast cells, especially when the percentage of the pathologic cells is low. At present, the association of angular light scatter (side scatter, SS) with an immunologic marker is the preferred method for blast cell identification.12,13,42 The single immunologic marker best suited for this purpose is CD45.

CD45 is a pan-leukocytic antigen which displays different patterns of expression with unique densities in the different normal peripheral major leukocyte subpopulations (Figure 1) being more intense in lymphocytes and monocytes than in granulocytes.

CD45 expression in blast cells is typically lower than in lymphocytes12,42 (Figure 1B). In our experience, this approach can be further strengthened by the use of additional lineage-associated antigens since the lineage-specific antigen will contribute to improving the SSC/CD45 gating strategy especially in bone marrow samples which may contain normal residual precursors.

Selection of reagents

In almost all cases assessment of antigen expression is based on the use of a combination of fluorochrome conjugated monoclonal antibodies (MoAb) and direct immunofluorescence techniques. At present clinical flow-cytometers are usually equipped with three or four fluorescence detectors in order to allow for the simultaneous analysis of up to five or six parameters; two light scatter (forward and side) and three or four immunofluorescence colors. Instruments equipped with four photo-multipliers can increase the light emission of up to 4 different fluorochromes either using two fluorochrome tandems [(R-phycocerythrin-Texas red (ECD) + R-phycocerythrin-cyanin 5 (R-PE/Cy5)] and a single blue (488 nm) laser (Becton Dickinson Coulter, Inc., Miami, FL, USA) or allophycocyanin (APC) and either peridinin-chlorophyll protein (PerCP) or the PerCP/Cy5 tandem using both a blue laser (488 nm) and a red diode laser (635 nm) (Becton Dickinson Biosciences, San José, CA, USA).

In order to select for the most adequate combinations of reagents, the investigator must know the characteristics of both the antigens and the fluorochromes. Accordingly, MoAb clones must be carefully selected since several clones recognizing the same CD may differ in their reactivity, as proven by the CD15 (glycosylated or not glycosylated form) and CD34 (class I, class II, class III) reagents.42,43

Variability between different batches of the reagents is not a major problem. However, it is recommended that every new vial is checked. Recent studies have demonstrated that intracellular antigens can currently be easily detected with different commercially available fixation/permeabilization kits. However, when selecting a particular fixation/permeabilization reagent its combination with particular antibody-conjugates should be tested.44,45

In general PE-conjugates are more sensitive than FITC ones while PE/Cy5 and APC reagents show an intermediate performance. Accordingly, the brightest fluorochrome, PE should be used for the weakest antibodies. The choice for a third and fourth fluo-
enumeration and characterization of blast cells

The diagnosis of acute leukemia is currently a multi-step procedure. Typically one of the first steps consists of microscopy examination of blood or bone marrow smears. The morphologic/cytological (FAB classification) examination of these smears still plays a relevant role. The presence of myeloperoxidase (MPO) and/or Sudan black or a strong/diffuse positivity for non-specific esterases (ANAE) in the blast cells represents an unequivocal sign of non-lymphoblastic origin of leukemia.12

Multiparametric (three or four color) flow cytometry immunophenotyping must be used for characterization of the cells present in the sample.

The major difference between two color immunophenotyping and the herein proposed analysis is related to the gating strategy. In two color analysis, gating is typically based on morphology-related parameters (i.e. light scattering parameters, FSC, SSC).11,12,22 In three/four color immunophenotyping one marker is usually used in association with orthogonal scatter (SS) for immunologic gating of the cells of interest. With the last method, the sensitivity and specificity achieved in the identification of blast cells is clearly higher.

Enumeration and characterization of blast cells

The number of pathologic cells in bone marrow or in peripheral blood is critical for diagnosis under various conditions. For example, according to the FAB and WHO classifications, the differential diagnoses of non-Hodgkin’s lymphomas and ALL or myelodysplastic syndromes (MDS) and acute myeloblastic leukemias (AML) are based on the percentage of blast cells in the bone marrow.12,14

Immunophenotypic characterization of blast cells has several goals: 1) lineage assignment; 2) evaluation of cell maturation; 3) assessment of phenotypic aberrations. At the same time, it provides information on the degree of heterogeneity of a leukemic cell population.

Adequate instrument set-up and calibration are essential for all these goals and especially for day-to-day comparison of results.

Lineage assignment of blast cells by immunophenotype still represents a major challenge in some acute leukemias. This is mainly due to the existence of cross-lineage antigen expression. Several antigens which were claimed, in normal samples, to be specific to or associated with lymphocytes and NK cells (CD2, 7, 19, 56), and myeloid cells (CD13, 14, 15, 33, 65) have now been reported as positive in a proportion of AML and ALL cases.18,23,26,29,35-40,46 These observations emphasize the need to use combinations of several lineage-associated markers in order to establish the lineage of blast cells. The choice must include antigens with high sensitivity that are fully present in a certain lineage (e.g. CD7 in T-cells or CD19 in B-cells) together with more specific markers (e.g. CD3 for T-cells, or MPO for AML). Additional progenitor cell
markers such as CD34 are used to confirm the immaturity of the pathologic cells (Table 1).

From the practical point of view, lineage assignment should typically be based on the combined use of markers for immaturity and for the different lymphoid (B, T) and myeloid lineages together with markers that allow the identification of blast cells. The CD7/CD34/CD19/CD45, cCD3/cyCD79a/CD34/CD45 and cMPO/CD33/CD34/CD45 combinations represent practical examples. Using the combination CD7/CD34/CD19/CD45, precursor B-ALLs result as being CD19 and CD34 positive. In the few CD34 cases the altered expression of CD45 (lower expression than normal B-lymphocytes) allows differentiation between the normal immature and the neoplastic B-cells (Figure 1 C,D,E,F).22 The precursor T-ALLs are CD7 positive and CD19 negative. The simultaneous absence or anomalous reactivity for CD19 and CD7 in the presence of positivity for other myeloid-associated markers supports a non-lymphoid origin of the leukemia even in the absence of MPO.46-49

Specific combinations could be used to monitor therapy efficacy and minimal residual disease.13, 22-25 Table 2 gives a list of the most useful antigens for immunophenotypic diagnosis of acute leukemias. Once lineage and maturation stage of the blast cells have been identified the role of immunophenotyping focuses on their subclassification through the identification of homogeneous clinically relevant subgroups.

Acute lymphoblastic leukemias
Specific phenotypic characterization of ALL blast cells is usually performed using lineage associated markers such as CD19 for B-cells and CD7 for T-blasts in all tube combinations. Other markers are used to identify the maturation level of the blast cells and eventually establish a typical or aberrant

figure 1. The four color combination CD7/CD34/CD19/CD45. Blast cell identification is obtained using the combination based on light scatter parameters and 2 antigens with high sensitivity (CD7, CD19) and 2 with high specificity (CD34, CD45). The A and B histograms demonstrate the different ability of either light scattered parameters (FS and SS log) or CD45 and light scatter (SS log) in identifying various bone marrow populations (lymphocytes, monocytes and blast cells). The combinations with CD7 and CD34 (C), CD19 and CD34 (D), permit an adequate identification of blast cells. The CD19 immunological gate (E) and combination CD10/CD20 (F) in CD19 gated cells.
phenotypes indicative of specific underlying genetic lesions.

Assessment of the blast cell maturation stage has proven to be of unequivocal clinical value. As an example differentiation of mature B-ALL cases from early B-cell ones is of great clinical relevance. B-ALL cases are characterized by translocations involving the c-Myc gene located on chromosome 8 q24 with three different partner chromosomes: t(8;14); t(2;8) and t(8;22). These genetic events are translated into a high proliferative rate (more than 30% S-phase) of tumor cells which, from the therapeutic point of view, benefit from more intense and shorter therapeutic protocols.

In various large studies the clinical relevance of myeloid-related antigens in ALL has been recently denied, at least in children with ALL, putting an end to the enormous amount of controversy in the literature.

The three/four color panel of reagents recently utilized in the AIEOP central laboratory for the phenotypic characterization of B-cell ALL is reported in Table 3. This panel is able to identify the major subtypes of B-cell ALL and prognostically relevant genotypes:

<table>
<thead>
<tr>
<th>Major subtypes of B origin ALL</th>
</tr>
</thead>
<tbody>
<tr>
<td>BI/ProB/Early B: (cyCD79a+), CD19−, HLA-DR+, Tdt+, CD10−, CD20+, cyIgm+, Sig−</td>
</tr>
<tr>
<td>BII/common/Early B: (cyCD79a+), CD19−, HLA-DR+, Tdt+, CD10+, CD20−/+ cyIgm+, Sig−</td>
</tr>
<tr>
<td>BIII/Pre B: (cyCD79a+), CD19−, HLA-DR+, Tdt+, CD10+, CD20−/+ cyIgm+, Sig−</td>
</tr>
<tr>
<td>BIV/B mature: (cyCD79a+), CD19−, HLA-DR+, Tdt−/+ , CD10−/+ CD20+, cyIgm+, Sig− (κ or λ −)</td>
</tr>
</tbody>
</table>

Finally, specific antibody combinations could be used in order to identify prognostically relevant genetic translocations. The identification of the genetic translocations is relevant for the correct stratification of patients into risk groups receiving different treatment protocols and also provides a sensitive and specific tool for minimal residual disease (MRD) follow-up. In acute lymphoblastic leukemia of B-cell origin it is possible to define homogeneous subgroups with different prognoses related to associated chromosomal abnormalities.

Table 1. Antigen utilization in the immunophenotyping of acute leukemias.

<table>
<thead>
<tr>
<th>Antigens with broad expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pan-myeloid</td>
</tr>
<tr>
<td>Pan-B-cell</td>
</tr>
<tr>
<td>Pan-T-cell</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Antigens associated with immaturity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pan-lymphoid and myeloid</td>
</tr>
<tr>
<td>Pan-myeloid</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Antigens with lineage-specific and maturation-dependent expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myeloid cells</td>
</tr>
<tr>
<td>Erytoid cells</td>
</tr>
<tr>
<td>Platelets</td>
</tr>
<tr>
<td>B-cells</td>
</tr>
<tr>
<td>T-cells</td>
</tr>
<tr>
<td>NK-cells</td>
</tr>
</tbody>
</table>

Cy: cytoplasmic, s: surface membrane.

A very good prognosis has been associated with t(12;21) (q13;q22) (TEL/AML1)55−57, 96,99 and hypodiploid DNA content. It has been demonstrated that this translocation occurs in a substantial subgroup of childhood leukemia whereas it is uncommon in adults. The immunophenotype is characterized by low or absent CD45 and CD20, high CD10, and bimodal CD34 expression.56,59

In contrast, poor prognosis has been associated with BCR/ABL and MLL/AF4 fusion genes – resulting from t(9;22) (q34;q11) and t(4;11) (q21;q23) translocations, respectively. Immunophenotyping shows specific CD34 and CD38 expression in t(9;22) translocations. The 11q23 translocations are characterized by positive antibody staining for NG2 and CD15 and negative for CD10.55,60,61,81,82

T-cell ALL

The CD7 marker shows a high specificity in T-ALL, in fact it is present in about 100% of patients. However the sensitivity is not absolute since this antigen can also be detected in around 20% of AM L63 cases. This renders this marker useful for blast cell gating strategies in combination with CD45 in T-ALL. Further characterization of the phenotype of T-ALL blast cells is aimed at subclassification into early T versus more mature T-cell ALL.

T-lineage ALL has been previously divided into three stages of immunophenotypic differentiation: early (CD7+, cCD3+, surface CD3+, CD4− and CD8−), intermediate (cCD3, surface CD3+, CD4+, CD8+) and...
CD1a+ and late (surface CD3+, CD1a−, and either CD4+ or CD8+).64

There is no general consensus about the clinical relevance of maturation levels for T-ALLs. Some groups have reported a better outcome for the intermediate CD1a positive subgroup64-67 while these data are not fully confirmed by other series in which the subclassification of T-ALL did not show clinical relevance.68-71 Furthermore controversial data have been reported about the prognostic significance of some antigens in T-ALL. For example a favorable significance has been associated with CD2,72-74 CD3, CD4, CD8, and CD10.75,76

<table>
<thead>
<tr>
<th>Designation</th>
<th>Cell type</th>
<th>Antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD1a</td>
<td>Cortical thymocytes, subpopulation of B-cells, dendritic cells</td>
<td>MHC-like protein, can associate with β2-microglobulin</td>
</tr>
<tr>
<td>CD2</td>
<td>T-cells, most NK cells</td>
<td>Erythrocyte-rosette receptor</td>
</tr>
<tr>
<td>CD3</td>
<td>Surface expression on mature T-cells, cytoplasmic</td>
<td>Associated with the T cell receptor, mediates signal expression in immature T cells transduction</td>
</tr>
<tr>
<td>CD4</td>
<td>Helper/inducer T-lymphocytes, monocytes</td>
<td>Receptor for MHC II molecules</td>
</tr>
<tr>
<td>CD5</td>
<td>Thymocytes, mature T-cells, subpopulation of B cells</td>
<td>Linked to T cell proliferation</td>
</tr>
<tr>
<td>CD7</td>
<td>T-cells, NK-cells, subpopulation of immature myeloid cells</td>
<td>40 kDa protein</td>
</tr>
<tr>
<td>CD8</td>
<td>Cytotoxic/suppressor T-cells, subpopulation of NK-cells</td>
<td>Receptor for MHC I molecules</td>
</tr>
<tr>
<td>CD10</td>
<td>c-ALL, lymphatic precursor cells, neutrophils subset of mature B cells</td>
<td>Common acute leukemia antigen (CALLA), neutral endopeptidase</td>
</tr>
<tr>
<td>CD11a</td>
<td>Monocytes, macrophages, neutrophils, NK-cells</td>
<td>Adhesion molecule, CD3 receptor</td>
</tr>
<tr>
<td>CD11c</td>
<td>Monocytes, neutrophils, NK-cells, subpopulation of B-cells</td>
<td>Adhesion molecule, gp 150/95</td>
</tr>
<tr>
<td>CD13</td>
<td>Myeloid cells</td>
<td>Aminopeptidase N</td>
</tr>
<tr>
<td>CD14</td>
<td>Monocytes and neutrophils</td>
<td>LPS receptor</td>
</tr>
<tr>
<td>CD15</td>
<td>Neutrophils and monocytes</td>
<td>X-hapten, 3-fucosyl-N-acetyl-lactosamine</td>
</tr>
<tr>
<td>CD16</td>
<td>NK-cells, neutrophils, subpopulation of monocytes</td>
<td>Low-affinity Fc-receptor for IgG</td>
</tr>
<tr>
<td>CD19</td>
<td>Precursor B-cells, B-cells</td>
<td>Bridge for surface immunoglobulin signal</td>
</tr>
<tr>
<td>CD20</td>
<td>Subpopulation of precursor B-cells, B-cells</td>
<td>Ion channel, protein kinase C substrate</td>
</tr>
<tr>
<td>CD22</td>
<td>Surface expression on B-cells, cytoplasmic expression in precursor B-cells</td>
<td>Related to neutral cell adhesion molecule, bridge for surface immunoglobulin signal</td>
</tr>
<tr>
<td>CD24</td>
<td>Monocytes, myeloid precursor cells, weak expression on dendritic cells</td>
<td>67–45 kDa glycoprotein</td>
</tr>
<tr>
<td>CD34</td>
<td>Myeloid and lymphoid precursor cells</td>
<td>105–120 kDa glycoprotein</td>
</tr>
<tr>
<td>CD38</td>
<td>Activated lymphocytes, subpopulation of B-cells, plasma</td>
<td>45 kDa glycoprotein</td>
</tr>
<tr>
<td>CD40</td>
<td>Platelets, megakaryocytes</td>
<td>Glycoprotein IIb/IIIa, fibrinogen receptor</td>
</tr>
<tr>
<td>CD41</td>
<td>Platelets, megakaryocytes</td>
<td>Glycoprotein IIb, part of the Willebrand factor receptor</td>
</tr>
<tr>
<td>CD42b</td>
<td>Platelets, megakaryocytes</td>
<td>Glycoprotein IIb, part of the Willebrand factor receptor</td>
</tr>
<tr>
<td>CD43</td>
<td>Actin</td>
<td>72 kDa antigen, protein-lysine-phosphatase</td>
</tr>
<tr>
<td>CD45</td>
<td>NK-cells</td>
<td>N-CAM</td>
</tr>
<tr>
<td>CD56</td>
<td>Platelets, megakaryocytes</td>
<td>Glycoprotein IIb, vittrein receptor β-chain</td>
</tr>
<tr>
<td>CD64</td>
<td>Monocytes, macrophages</td>
<td>High-affinity receptor for IgG</td>
</tr>
<tr>
<td>CD65</td>
<td>Neutrophils, weak expression on monocytes</td>
<td>Carbohydrate-dodecasaccharide 40</td>
</tr>
<tr>
<td>CD66c</td>
<td>Neutrophils</td>
<td>Glycosyl-phospholipidinositol (GPI) linked</td>
</tr>
<tr>
<td>CD68</td>
<td>Monocytes</td>
<td>GP 110</td>
</tr>
<tr>
<td>CD71</td>
<td>Erythroid cells, activated T- and B-lymphocytes, macrophages</td>
<td>Transferrin receptor macrophages</td>
</tr>
<tr>
<td>CD79b</td>
<td>B-lymphocytes, including immature B-cells</td>
<td>Igα mb3, part of the B-cell antigen receptor</td>
</tr>
<tr>
<td>CD80</td>
<td>B-lymphocytes, activated T-lymphocytes, monocytes</td>
<td>67 antigen, glycoprophosphatidylinositol-linked glycoprotein</td>
</tr>
<tr>
<td>CD83</td>
<td>Dendritic cells</td>
<td>43 kDa glycoprotein</td>
</tr>
<tr>
<td>CD95</td>
<td>Mature and immature cells</td>
<td>Fas</td>
</tr>
<tr>
<td>CD116</td>
<td>Myeloid cells</td>
<td>GM-CSF receptor α chain</td>
</tr>
<tr>
<td>CD117</td>
<td>Myeloid precursor cells</td>
<td>CDK, stem cell factor receptor</td>
</tr>
<tr>
<td>CD133</td>
<td>Precursor myeloid and lymphoid cells</td>
<td>FLK2/FLT3</td>
</tr>
<tr>
<td>Glycoporphin A</td>
<td>Erythrocytes, erythroblasts and erythroid precursor cells</td>
<td>Sialic acid-rich polypeptide</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>B-lymphocytes, activated T-lymphocytes, monocytes,</td>
<td>Part of the HLA class II complex precursor cells</td>
</tr>
<tr>
<td>MPO</td>
<td>Lysosomal expression in neutrophils and monocytes,</td>
<td>Myeloperoxidase including immature myeloid cells</td>
</tr>
<tr>
<td>Lactoferrin</td>
<td>Neutrophils and monocytes</td>
<td>Lactoferrin</td>
</tr>
<tr>
<td>TdT</td>
<td>Nuclear expression in lymphoid precursor cells</td>
<td>Terminal deoxynucleotidyl transferase</td>
</tr>
<tr>
<td>TCR αβ</td>
<td>Majority of T-lymphocytes</td>
<td>ε/β chains of T-cell receptor</td>
</tr>
<tr>
<td>TCR γδ</td>
<td>Subset of T-lymphocytes</td>
<td>γ/δ chains of T-cell receptor</td>
</tr>
<tr>
<td>IgM</td>
<td>Surface expression on B-lymphocytes</td>
<td>Immunoglobulin-heavy chain type α</td>
</tr>
<tr>
<td>IgG</td>
<td>Surface expression on B-lymphocytes</td>
<td>Immunoglobulin-heavy chain type α</td>
</tr>
<tr>
<td>IgA</td>
<td>Surface expression on B-lymphocytes</td>
<td>Immunoglobulin-heavy chain type α</td>
</tr>
</tbody>
</table>
gen expression) have not been unequivocally con-

confirmed.67, 70,74,76 These results are not clinically use-

ful, and indeed they are not included in stratifica-

tion for treatment. Some groups, including AIEOP,

BFM and CCSG70,76,77 are putting major emphasis

on early good response to therapy as the best pre-

dictor of outcome in T-ALL.

A possible potentially useful panel of monoclon-

al antibody combinations for the characterization

of T-ALL is shown in Table 4.

Acute myeloid leukemia

From both diagnostic and clinical points of view,

three different major groups should be established

among non-lymphoblastic acute leukemias: mini-

mally differentiated (MPO-)AML, acute promyelo-

cytic leukemia (APL) and the other AML cases.

A rare AML subtype is constituted by the mini-

mally differentiated MPO negative AML. This sub-

group is characterized by being of myeloid origin

without evidence (less than 3%) of MPO in flow

cytometry or in cytochemistry and negativity for

ANAE. The immunophenotype of these cases is

characterized by the absence of expression of high-

ly specific lymphoid markers (i.e. CD3, cyCD79a)

and by reactivity for myeloid related antigens

(CD13, CD33 and/or CD117) and for CD7 and CD34

(Figure 2 D,E,F).11,47-49 These leukemias are usually

heterogeneous both from the clinical/cytogenet-

ic
and prognostic points of view as well as with respect to their light scattering and phenotypic properties.

More differentiated AML cases

For a long time the FAB classification has been the most widely accepted set of criteria for classifying acute non-lymphoid leukemias. The correspondence between FAB subclasses and some specific genetic aberrations reinforces the relevance of conventional morphologic criteria. The role of immunophenotyping in the subclassification of AML is still under discussion although general agreement exists on its relevance to the diagnosis of M0, M6 and M7 subtypes.

In spite of this, in recent years multiparametric immunophenotyping has shown that some specific genetic aberrations are associated with unique patterns of antigen expression. Furthermore, in some instances flow cytometry provides a more accurate enumeration of the blast cells than do morphological criteria. Immunophenotyping may be of help in areas such as the differential diagnosis between AML and myelodysplastic syndromes (MDS) and the subclassification of these latter. As opposed to what happens in precursor-B or T-ALL, the blast cells in AML are usually more heterogeneous and more than one cell lineage can be involved.

AML represents a group of leukemias composed by less (M0, M1, M5a) or more (M2, M3, M5b) differentiated diseases and by single (M1, M3, M5) or mixed (M4, M6, M7) cell lineage leukemic blasts. The greater heterogeneity of non-lymphoid blast cells in the bone marrow may have an impact on the ability to identify blast cells precisely. At the same time, exclusive evaluation of typical blast cells could be misleading since leukemic myeloid cells frequently retain a certain ability to mature. The identification and interpretation of the differentiation pathways of myeloblasts represent major challenges for the classification of AML.

This supports the relevance of minimizing manipulation of samples during sample preparation or data acquisition/analysis.

Based on this, flow cytometric multiparametric
analysis of AML should deal first with the identification of the pathologic myeloid cells as opposed to the normal cells present in the sample. Assessment of the potential involvement of mature cell populations should be done in a second step. Finally, the maturation pathways of the pathologic cells should be identified based on tumor heterogeneity for antigen expression.

At present consensus exists on the choice of the CD45 immunologic marker for gating purposes in AML, although it is well-known that a single marker for all possible myeloid leukemic populations does not exist. CD45 should be used in association with light scatter; we have found this to be adequate in gating the majority of AMls studied. In some cases CD45 is not suitable for gating purposes because of the marked heterogeneity of bone marrow cells or the limited number of blasts present in the sample. In such cases CD13, CD34 or CD34 may be of help.

Some antigen combinations which might be of great utility for the follow-up of monocytic and granulocytic differentiation include SSC/CD14/CD64/CD45/CD34 and SSC/CD15/CD16/CD45/CD65, respectively. In spite of the similarities between leukemic and normal myeloid cells, the existence of atypical antigen expression is currently considered a useful finding for specific identification of leukemic cells. In recent years it has been shown that, similarly to ALL, the patterns of leukemic-associated antigen expression correlate with specific translocations. These observations together with the production of a new set of reagents against fusion proteins or proteins expressed in cells carrying a specific translocation have provided new insights into the importance of immunophenotyping in the classification of AML. The identification of three subtypes of AML has been critical for prognosis-based treatment. A diagnosis of M3 (t(15;17)) implies a specific therapeutic program involving the AML1 and ETO genes in chromosomes 21 and 8, respectively. From the genetic point of view it has been suggested that in AML this genotype is associated with a higher sensitivity to therapy and a better outcome.

Acute myeloblastic leukemia with granulocytic differentiation and t(8;21)

The M2 leukemia with t(8;21) is a unique subgroup of M2 cases characterized by a balanced translocation involving the AML1 and ETO genes in chromosomes 21 and 8, respectively. The clinical point of view has been suggested that in AML this genotype is associated with a higher sensitivity to therapy and a better outcome.

Acute myelomonocytic leukemia with eosinophilia (M4EO)

Morphologically this myelomonocytic AML subtype is characterized by coarse, densely-staining granules in the eosinophils, with a high percentage of these cells in the smear. From the genetic point of view inversion or translocation of chromosome 16 [inv16 or t(16;16)] involving the CBFβ-MYH11 genes is typical of these cases. Clinically this chromosomal abnormality has been associated with a more favorable prognosis. In a high percentage of cases, immunophenotypic studies show co-expression of CD2, CD4, CD7, CD13, CD14, CD15, CD33, CD34, CD64, CD65, CD117 and HLA-DR in the blast cells.

Immunophenotyping of other AML cases not genetically classifiable

The most important point regarding the immunophenotypic characterization of these AML cases is the evaluation of the degree of differentiation and the identification of aberrant phenotypes. Within this subgroup, the identification of AML cases displaying similar patterns of antigen expression will in the near future certainly contribute to a clinically relevant subclassification of these patients, especially among M2, M4 and M5 (clin-
cally relevant) leukemias. Assessment of megakaryocytic or erythroid lineage involvement through the expression of megakaryocytic [CD41a, CD42b, and CD61] and erythroid [glycophorin A, CD36, CD71++] related markers is of great utility for the identification of these subtypes of AML. However, reactivity for these antigens must be examined with care since the possible non-specific coincidence of platelets or red cell membrane fragments with the blast cells during analysis may lead to misclassification.

Although expression of some antigens has been correlated with prognosis (i.e. CD7 and CD34, CD56), controversial results exist in the literature concerning this subject. Recently in adult de novo AML, pan-myeloid phenotype (defined by the full expression of MPO, CD13, CD33, CDw65 and CD117), performance status, and permeability glycoprotein activity have been demonstrated to influence treatment outcome with a high prognostic impact in a multivariate analysis. These results are very interesting even if not confirmed by following series and despite the employed analysis criteria not having been adequate. The back-bone of the analysis, that a pattern of various antigens and not single antibody positivity could be relevant for the prognosis, is shared. Extensive studies with a multiparametric quantitative approach may increase the importance of some myeloid antigens in identifying homogeneous AML subtypes with prognostic significance. This approach overlaps the results obtained using traditional studies with 20% criteria of positivity.

A possible potentially useful panel of monoclonal antibody combinations for the characterization of AML is shown in Table 5.

### Acute biphenotypic leukemia

A certain number of acute leukemias have blast cells that simultaneously demonstrate features characteristic of both the myeloid and lymphoid lineages and for this reason are designated mixed-lineage, hybrid or biphenotypic acute leukemias (BAL). The real incidence of this form of leukemia varies considerably between different studies probably because of various technical aspects (antigen studied and gating strategy criteria). There are no widely accepted criteria for defining such leukemias and there are difficulties in establishing whether they represent a distinct clinical and biological entity. Probably the real biphenotypic leukemias are rare entities originating from a primitive stem cell with the potential to differentiate along the lymphoid or myeloid lineage.

#### Table 5. Possible antibody combination for myeloid AML characterization.

<table>
<thead>
<tr>
<th>CD7 POSITIVE BLASTS</th>
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<th>R-PE</th>
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<tr>
<td>CD65</td>
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<td>CD7</td>
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<td>CD65</td>
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Beckmann-Coulter instruments: third color PE-Cy5 or ECD. Becton-Dickinson instruments: third color PerCP or PE-Cy5.

The scoring system devised by the European Group for the Immunological Classification of Leukemia (EGIL) based on the number and degree of specificity of the markers (lymphoid and myeloid) expressed by the blasts is not fully adequate for identification of these forms. We considered as hybrid a leukemia characterized by positive myeloperoxidase (more than 3% in the blast cells) and a complete lymphoid immunophenotype with or without myeloid antigen expression. Positive myeloid antigens in ALL is not a criterion for diagnosing hybrid leukemia. The high frequency of myeloid antigen positivity in t(12;21)
and in t(9;22) ALL confirms this. Myeloid-associated antigens have no prognostic significance in childhood ALL subtypes.\textsuperscript{51-52,106,107} Different results have been reported in adult myeloid antigen positive ALL, in which a poorer response to standard ALL therapy has been reported.\textsuperscript{108} Since these phenotypes are frequently associated with molecular genetic abnormalities, the data related to poor prognosis could be explained by frequencies of prognostically relevant chromosome abnormalities in childhood [t(12;21)] and in adults [t(9;22)].

Acute leukemias with other phenotypes
The disease spectrum of rare forms of leukemias such as natural killer (NK) cell leukemias has been continuously expanding. Acute leukemia of NK-cell lineage is a very rare disease.

Suzuki et al.\textsuperscript{108} described seven cases of acute leukemia of myeloid and NK cell precursor phenotype with strikingly extramedullary involvement at presentation. The expression of CD7, CD33, CD34, CD56 and frequently HLA-DR, but not MPO nor other NK, T-cell and B-cell markers was observed.\textsuperscript{109,110}

Most reports suggest that this form of acute leukemia, most common in adults but described also in childhood,\textsuperscript{107} may arise from the transformation of a precursor cell common to the NK-cell lineage and myeloid or T-lineage.\textsuperscript{109,111-113}

Its recognition appears to be particularly important for the clinical pathologic evaluation of CD56+ hematolymphoid malignancies and for the development of therapeutic approaches to such diseases with an aggressive outcome.\textsuperscript{108} The T-cell receptor DNA rearrangement demonstrated in these forms makes the NK cell origin of these leukemias doubtful. Furthermore CD56 is positive in a few CD19+ ALLs and these leukemias are not classified as NK leukemias.

A dendritic cell leukemia has been included in the WHO classification.\textsuperscript{14} No cases with principal and exclusive bone marrow involvement have yet been described in the literature. In our experience only one case has been classified as acute dendritic leukemia. The diagnosis was made by characteristic morphology and by immunophenotyping showing positivity for CD83 and CD86.

Criteria for positivity and quantitative evaluation of antigen expression
Flow cytometry immunophenotyping allows qualitative and quantitative evaluation of antigen expression by individual cells. The analysis of high numbers of events provides patterns of antigen expression for leukemic cell populations. Accordingly, apart from the assessment of the presence/absence of an antigen, information is provided on the amount of antigen expressed/cell and the pattern - homogeneous versus heterogeneous - of antigen reactivity. Thus, quantitative analysis of antigen expression is essential and is very useful for the differentiation between normal and leukemic cells as well as for the identification of different subtypes of AL with a similar genetic background.\textsuperscript{94-98}

Antigen expression should therefore be evaluated by the mean fluorescence intensity and coefficient of variation found for the leukemic blasts as opposed to the frequently used cut-off values of positive cells (typically more than 20% positive cells). We consider a leukemic cell population to be positive for a specific antigen when the median fluorescence intensity (MFI) found for this antigen is higher than that obtained for the negative cells (unstained cells) plus 2 standard deviations (SD).

Antigen intensity (MFI) can be expressed in several different units including MESF or ABC (antibody binding capacity) depending on the operative system used. The use of standardized units is usually preferred for obtaining reproducible results at either different time points in the same laboratory and/or in different laboratories. Fluorescence quantification in standardized units such as MESF is typically obtained through instrument calibration with a set of fluorescent standard beads with different well-known amounts of fluorochrome. The peak channel distribution for each of the different beads (1=negative) obtained in the fluorescence histogram is plotted against the known value of molecules of soluble fluorochrome (MESF) for each population of beads in order to provide a standard curve.

As mentioned earlier, antigen quantification has proven of use in the identification of specific prognostic subtypes of both ALL [i.e. t(12,21)+, t(9,22)+] and AML [i.e. t(15;17)]. Accordingly, the use of quantitative multiparametric immunophenotyping techniques for a more profound analysis of the patterns of expression of antigens on blast cells has been reported. The t(12;21) in precursor B-ALL is identifiable with high sensitivity (87%) and specificity (100%) by high expression of CD10, HLA-DR, and by low expression or absence of CD20 and CD135 and by bimodal expression of CD34.\textsuperscript{99} A unique pattern of CD38, CD34, CD13, and CD10 has been demonstrated in adults with t(9;22)+ precursor B-ALL.\textsuperscript{62} The APL with t(15;17) shows a typical phenotype with regard to CD13, CD34 and CD15 expression which allows highly specific and sensitive identification of this AML subgroup.\textsuperscript{99} The t(4;11) ALLs characteristically show CD45, CD15, CD19, NG2 positivity, and are usually CD10 negative.
Minimal residual disease

Common therapeutic protocols have considered 5% of blasts cells in the BM as the morphologic limit for considering the patient in complete remission (CR). More recent studies using molecular and immunophenotypic methods have demonstrated the presence of quantifiable disease in patients considered in morphologic CR. The studies of minimal residual disease (MRD) should improve the estimate of the number of blast cells present in the bone marrow in CR during and after therapy thus improving the clinical management of patients with acute leukemia. MRD studies involve early detection of relapse and evaluation of drug efficacy by estimating the in vivo clearance of blast cells. MRD studies are also of potential utility in the setting of autologous stem cell transplantation in order to evaluate the possible presence and levels of blast cells in the harvested sample.

Numerous methods including cytogenetic, molecular genetic and immunologic approaches have been developed over the last decade aimed at the sensitive detection of MRD. The cytogenetic methods use karyotypic abnormalities, defined at diagnosis, during follow-up either after cell culture or by interphase fluorescent in situ hybridization (FISH) to identify the blast cells. This approach has a high specificity but its sensitivity is relatively low. In fact, in the best conditions, 1/1000 cells have been identified. Molecular approaches are usually based on PCR technology to identify, with high specificity and sensitivity (10−5/10−6 cells), either fusion products of translocations (BCR-ABL, E2A-PBX1, MLL-AF4, TEL-AMLL1, AML1-ETO, PML-RARα) or specific B- and T-cell antigen receptor-gene rearrangements. Identification of fusion products of translocations is a simple, fast and cheap but only about 30% of ALL and 25-30% of AML can be monitored in this way. Molecular approaches are usually based on PCR technology to identify, with high specificity and sensitivity (10−5/10−6 cells), either fusion products of translocations (BCR-ABL, E2A-PBX1, MLL-AF4, TEL-AMLL1, AML1-ETO, PML-RARα) or specific B- and T-cell antigen receptor-gene rearrangements. The second approach is based on the existence of abnormal patterns of antigen expressions in the blast cells with respect to the pattern of the same antigens in normal bone marrow cells (Figure 3). The latter approach utilizes either the abnormal higher expression of CD10, CD38 and CD58 or the lower expression of others such as CD45 and CD11a in the ALL of B-cell origin. In the last methodology the empty space approach has been suggested by Biomed concerted action and by Borowitz. According to this methodology, a series of three color or four color combinations have been employed to design a map of the normal pattern of B-cell differentiation. The blast cells are identified by their aberrant position in the dot plot. This methodology is very interesting but the MRD studies are performed in treated patients in whom bone marrow is not normal but regenerating or hypoplastic. The pattern of regenerating B-cells is therapy-dependent as demonstrated by the Dutch group. For these reasons the comparative phenotype mapping based on identifying the specific dot plot of blast cells at diagnosis and in the follow-up could make an interesting, strong methodology to increase sensitivity and specificity during the follow-up. In any case the strategy to increase the sensitivity of the immunologic detection of MRD consists in acquiring only cells from a specific cell lineage — that of the blasts. This is achieved by using a specific immunologic gate in the acquisition or in the analysis. Thus, a high level of sensitivity can be obtained by screening more than 10⁶ or even 10⁷ cells without problems with the computer hardware. The presence of residual blast cells is confirmed once a homogeneous (for...
antigen expression and light scatter characteristics) group of cells (a cluster) with a leukemic phenotype is identified (at least 10^15 events would be necessary). Usually, detection of minimal residual disease in T-ALL by flow cytometry is relatively simple. In fact, the association of terminal deoxynucleotidyl transferase (TdT) and CD3 or CD5 represented in more than 90% of T-ALL is not present in normal bone marrow. The test sensitivity is high, around 10^-5. In the precursor B-ALL a more convenient approach consists in using a back-bone combination, such as CD10 /CD19 /CD34 (useful in 80% of ALL), to which a fourth antigen is associated with anomalous expression at diagnosis. Useful antigens were CD20 or CD45 or CD58 or CD66c or one positive myeloid antigen at diagnosis. Immuno-phenotyping is able to monitor MRD in about 80% of all AML, which compares with the 30% using molecular techniques. Various combinations have been reported in AML monitoring using the identical ALL approach. The great advantage consists in a high number of atypical expression antigens. The back-bone CD34/CD45 combination (utilized as the gate) associated with two positive antigens offers a high sensitivity and specificity in MRD (Figure 2 D,E,F). Previous reports have confirmed the clinical value of MRD monitoring of both ALL and AML patients as far as regards relapse prediction. An additional clinical value of the MRD approach is the confirmation of lymphocyte regeneration in the bone marrow after the therapy.

Apoptosis and multi-drug resistance

Some new apoptosis-related molecules such as CD95 (Fas/APO-1) and Bcl-2 are important factors for tumor cell survival and resistance to chemotherapy in hematologic malignancies. The role of the expression of these molecules is controversial in AL. The role of these molecules appears to be more relevant in AML than in ALL. Several studies have demonstrated a correlation between constitutive Bcl-2 expression and response to chemotherapy in AML. Only one study revealed a correlation between Bcl-2 expression levels and response to chemotherapy in ALL. Other studies could not confirm this finding. Constitutive CD95 expression and function in AML have been correlated with response to induction chemotherapy; these data are not confirmed in ALL.

One of the best-characterized resistance mechanisms is drug expulsion by P-glycoprotein (Pgp), the product of multidrug-resistance-1 (MDR1) gene. This mechanism contributes to chemotherapy failure in acute leukemia. Its expression level and its functional activity have been demonstrated to have a prognostic impact in adult AML but not in childhood ALL. Quantification of resistance will yield new, relevant information about this mechanism.

Conclusions

In summary the immunophenotypic analysis of leukemic cells provides essential information for the diagnosis and the follow-up of AL and this information should be combined with that of morphology and genetic investigations. Furthermore, the introduction and diffusion of new reagents specific for abnormal fusion products and antigen quantification will provide us with new tools for such investigations and the management of AL.

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