Recommended reporting format for flow cytometry diagnosis of acute leukemia

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A B S T R A C T

Although flow cytometry is increasingly used as a tool to diagnose hematologic malignancies, the reporting format of acute leukemia immunodiagnosis is still imprecise and sometimes vague, often reflecting old guidelines. Thus, the purpose of the present work was to make the reporting format for the immunological diagnosis of acute leukaemia easy and clear to understand. This work represents part of a more articulated series of technical guidelines that the Italian Society for Cytometry (GIC) is currently processing. Thirteen separate recommendations, covering all aspects of an acute leukemia cytometry report, are listed. According to our suggestions, the report must contain clear statements about: 1. demographic identification of patient; 2. identification of the hospital or division sending the sample; 3. type of specimen (bone marrow aspirate, peripheral blood, other biological fluids); 4. timing of observation (first diagnosis or follow-up); 5. diagnostic hypothesis made by the sender; 6. list of antigens and type of immunofluorescence analysis carried out; 7. absolute number of cells in the sample; 8. quality of the sample, in terms of viability; 9. general description of the gating procedure; 10. immunophenotype of blast cells; 11. description of cells surrounding blasts; 12. diagnostic conclusions; 13. definition of an antigen panel (when applicable) for the detection of minimal residual disease. As an example of a final report we present a case of acute myeloid leukaemia with t(8;21) translocation; in filling this report, we followed all the 13 points of the checklist described in the paper.

Key words: acute leukemia, reporting format, flow cytometry.

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Despite the profound changes already under way in the health care system, advances in biotechnology are increasingly ensuring that laboratory investigation will dominate the practice of medicine in the near future.[1] However, as a matter of routine, laboratory results need to be translated into information the clinician can understand. In this field, great effort has been made by Anatomic and Clinical Pathologists, who feel that the communication between laboratory staff and the clinician through the final report is an emerging issue which needs to be assessed.[2-4] Flow cytometry (FCM) is increasingly used as a tool to diagnose hematologic malignancies, and the goal of rendering the reports clear and understandable is a necessity.

The Italian Society for Cytometry (GIC) appointed several committees to develop recommendations about the content of clinical reports for common hematopoietic tumors. A working party comprised of seven members, selected on the basis of a special interest and expertise in immunodiagnostic technologies and procedures, produced these recommendations, which represent part of a broader series of technical guidelines that the Italian Society for Cytometry/GIC is currently processing. The purpose of the present work is to make the reporting format for the immunological diagnosis of acute leukemia easy and clear to understand.

Flow cytometry offers a series of powerful tools for precise definition of cell populations in bone marrow or peripheral blood specimens. Furthermore, by identifying sequential steps of cell maturation, flow cytometry is able to define different patterns of differentiation of individual lineages. As

an example, it is now possible to accurately identify the maturation pattern of B lymphocyte development in the bone marrow microenvironment.[5] In spite of these encouraging advances, the reporting format of immunodiagnostic information for acute leukemia is still imprecise and sometimes vague, often reflecting old guidelines. For instance, many FCM users are accustomed to simplistic data analysis approaches, such as *set quadrants and calculate the percentage of positive cells*, which may be inadequate in the complex process of leukemia immunophenotyping. The following are still unresolved questions: how to gate blast cells (i.e., by physical parameters, CD45 versus side scatter or other immunological gating strategies);[6,7] whether it is appropriate or not to report the percentages of positive cells for any antigen studied;[8] how to use the findings derived from fluorescence intensity analysis and how to express cell antigen density in the report.[9]

Features recommended for inclusion in the final report

Thirteen separate recommendations, covering all aspects of an acute leukemia cytometry report, are listed here.

Demographics. It is mandatory to record information that can identify patients precisely. This can be crucial not only at the time of diagnosis, but also during follow-up analyses, when the correct identification of the patient is a prerequisite. First name, last name, and date of birth of the patient, laboratory internal case number and Hospital codes must all be recorded.

Hospital or Division sending the sample. The report should always include a reference to the Institution which has sent the sample. If the flow cytometry laboratory belongs to a structure other than that sending the sample, it is mandatory to establish a cooperative relationship with the sender in order to obtain, either by phone, fax or e-mail, any and all information useful for a correct approach to immunodiagnosis and therapy. It is recommended that a comprehensive laboratory consultation service is provided. The complexity of medical technology demands it, and the primary care clinician needs it.[4] The cytometrist has a major consulting role in the diagnostic phase of clinical decision-making as well as in follow-up of controls.

Type of specimen (bone marrow aspirate, peripheral blood, other biological fluids). Specific reference to the tissue analyzed should be provided. The content of the report and technical procedures may be conditioned by the type of specimen. In the case of bone marrow and organic fluids, particular attention should be paid to the presence of mature cells surrounding the blast cell population (*see below*).

First observation or follow-up. It should always be stated in the report whether the sample has been taken at first diagnosis or during the follow-up. If the report refers to a follow-up control, previous results must be considered when formulating conclusions. In the case of a follow-up control, the cytometry laboratory will decide when to use (i) a complete diagnostic panel (e.g. in the presence of overt relapse), (ii) a smaller scheme able to confirm or deny remission status, or to evaluate the percentage of blast cells, (iii) a multicolor minimal residual disease approach, by counting high numbers of cells and searching for leukemia-specific phenotypes.

Diagnostic hypothesis. This is the clinical suspicion formulated by the people sending the sample when only incomplete information is available. Sometimes the diagnostic hypothesis is either vague or not confirmed by immunophenotyping analysis. An example of frequent discrepancies between pre-analytical hypothesis and post-analytical conclusion is differential diagnosis of acute lymphoblastic leukemia (ALL) versus immature acute myeloblastic leukemia (AML).

List of antigens and type of analysis carried out (three or four-color immunofluorescence). It is recommended that the antigens studied are listed carefully. Moreover, the location of the antigen studied (surface membrane or within the cell interior) should be reported. This rule applies only to molecules that can be expressed both on the membrane and in the cytoplasm (e.g. CD3, CD79a); when referring to internal staining,[10] it is sufficient to write cCD3 or cCD79a. The report will not include comments on individual antigens: findings that are irrelevant to diagnostic purposes should be avoided. Thus, antigens present in the list but not mentioned in the report must be considered irrelevant to the final diagnosis. It is also recommended that the laboratory uses the maximum number of colors feasible without compromising the reliability of the test, and reports the technology used (e.g., three-color or four-color immunofluorescence). At present, three-color typing should be considered the minimum technical requirement for analysis, whereas four-color analysis is the gold standard for high-quality immunophenotyping.[5] In the near future, it will be possible to perform clinically oriented five- and six- color large-scale analyses.[11]

Absolute number of cells. It is recommended that automated counts of nucleated cells are carried out with an electronic counter.[12] The goal is two-fold: first, since correct immunophenotyping needs to avoid problems due to excess antigen, overrich specimens, notably marrow aspirates, must be diluted before the immunostaining; second, the bone marrow cell count may provide valuable information on the hypo- or

hyperplastic status of bone marrow compartments. A final report should include a sentence referring to the amount of cells present in the sample (e.g. *bone marrow aspirate is rich in cells*, $50\times10^3/\mu L$). This is likely to be crucial for the interpretation of data; for instance, the detection of a unique population of $CD3^+$ lymphoid cells in the context of a very poor bone marrow indicates an aplastic process, while the finding of a unique T-cell population in a bone marrow massively infiltrated by lymphoid cells strongly suggests a T-cell lymphoproliferative disorder.[13] The presence/absence of bone marrow particles may be specified in the report, to give an idea of the contribution of dissociated bone marrow tissue to the overall cell number.

The report may include information from the senders about the status of a marrow sample, such as *the bone marrow aspiration was difficult or not* or *dilution by peripheral blood occurred during aspiration*. Moreover, cytometrists may confirm the quality of the marrow samples by seeing the amount of cells which usually exist preferentially in peripheral blood. For instance, a high percentage of mature lymphocytes in a bone marrow sample from a patient with acute leukemia suggests blood dilution.

Quality of the sample (viability, preservation status). It is recommended that the report provides mention of the status of cell viability and the cytographic integrity of the sample. It should be remembered that physical parameters are very sensitive indicators of the viability of hematopoietic cells in the sample.[14]

General description of the results of the gating procedure. The analytical point to be considered mandatory in cytometric analysis of acute leukemia is CD45 versus side scatter (SSC). The report must contain precise statements about the presence/absence of blast cells. It is often possible to assess the exact percentage of blast cells on the basis of CD45 vs SSC analysis. In some cases, it can be useful to use other immunological approaches to assess the percentage of blast cells accurately. In particular, CD7 and CD19, combined with CD45 are able to identify T-lymphoid and B-lymphoid blast cells, respectively.[6,7] Different conditions may be verified following the gating procedure of abnormal cells, and can ultimately lead to a restricted spectrum of mutually exclusive reporting sentences: a) blasts quantitatively dominate the cell suspension; b) blasts are clearly detectable admixed with normal and/or dysplastic accompanying cells; c) blasts cannot be detected by the preliminary gating strategy. One of these sentences must be provided in the report. The goal of this statement is not to anticipate diagnostic conclusions but to state clearly whether or not the specimen is informative (concerning the presence/absence of blasts). At the time of diagnosis, conditions defined by the first two statements (a or b) will enable a complete immunophenotyping. By contrast, the third condition is hardly compatible with a diagnosis of acute leukemia. During follow-up, sentences a and b will indicate leukemia relapse or resistance to therapy, while condition c will require further studies in terms of minimal residual disease analysis.

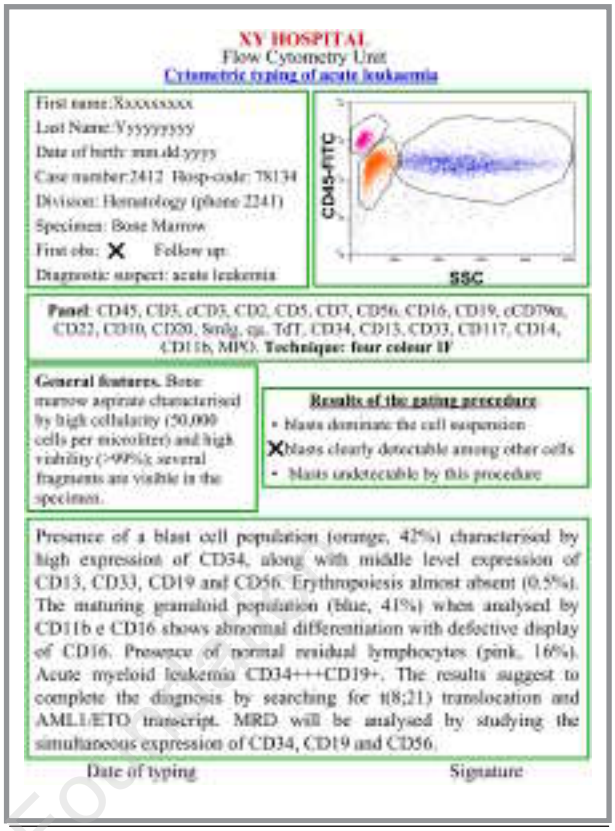
Immunophenotype of blast cells. The central point deals with reporting the exact percentage of blast cells (assessed in the gating procedure, see previous point) and qualitatively describing their immunophenotype (i.e., which antigens are positive and which are negative). Then the fluorescence intensity should be qualitatively reported for each relevant positive marker.[15,16] Different suitable analytical methods can be used in order to assess fluorescence intensity.[9] However, it is necessary to translate the information derived from mean fluorescence intensity (MFI), molecules of equivalent of soluble fluorochrome (MESF) or antibody binding capacity (ABC) calculations into a clinically understandable language. Very simple sentences should be used and numbers should be avoided. As an example: *blast cells express CD34, CD19, CD10 and CD22. The expression of CD10 is particularly high while CD19 and CD22 are expressed at a middle level intensity*. The clinical relevance of documenting expression intensity has been demonstrated: in pediatric B-ALL, high expression of CD10 and HLA-DR, along with low expression or absence of CD20 and CD135 and bimodal expression of CD34 is associated with good prognosis and t(12;21) abnormality.[6,17] By contrast, t(4;11) ALL, characterized by a worse prognosis, shows very low expression or, in most cases, absence of CD10, accompanied by NG2 positivity and low expression of CD15.[6,18] As for the definition of low, middle and high fluorescence intensity, we suggest that antigen expression should be considered low when its histogram is significantly different but not easily separable from the negative control histogram. Middle intensity is observed when the fluorescence intensity peak is contiguous to the negative control, but completely distinguishable from it. High expression intensity is present when the fluorescence peak is two or three logarithmic decades higher than that of the negative control. When considering fluorescence intensity, the cytometrist should distinguish relevant information, necessary for understanding the case, from irrelevant information. Reference should be made to heterogeneity or homogeneity of the staining. As an example: *CD34 is expressed in a bimodal way (low and high fluorescence intensity), testifying to the presence of a sub-population of leukemic cells characterized by an immature phenotype*. In order to classify a case of B-ALL correctly, bimodal expression of CD34 could help to define a clinically useful phenotype more completely.[6]

Various guidelines and classification systems have been published.[6,19,20] They tend to divide ALL and AML into subtypes on the basis of maturation stage. It must be remembered that nowadays, along with cytometric immunophenotyping, cytogenetic and molecular genetic analyses are standard in patients with newly diagnosed acute leukemia.

Description of cells surrounding the blast cell population. In addition to information on blast cells, it is essential in acute leukemia to describe the cells surrounding the blast population. As an example, an increased percentage of eosinophils may suggest an AML with inv16.[21] An increased percentage of erythroblasts may indicate the diagnosis of erythroid AML.[22] Moreover, an accurate description of a dysplastic background can be very useful.[23]

Diagnostic conclusions. It is always recommended that a diagnostic conclusion is given, when applicable. If used with expertise and caution, cytometry is the most powerful tool for differentiating AML from ALL. Therefore, this is the first endpoint which must be pursued. Immunophenotyping is the most powerful method to diagnose AML M0 (presence of myeloid markers along with lymphoid-like morphology and myeloperoxidase negativity) and AML M7 (expression of platelet antigens).[6] The second point is to define the maturation stage. For example, in the case of an AML it must be stated whether blast cells have a tendency towards maturation or not, and if they are committed towards granulocytopoiesis or monocytopoiesis. This information is very simply acquired from visual inspection of the CD45 versus SSC cytogram.[7] The maturation stage of B-ALL blasts is particularly relevant since pro-B, pre-B and B-cell acute leukemias are characterized by different genotypic patterns and clinical behaviors, often needing different treatment.[6,17,18] The third point is to use information about maturing cells surrounding leukemic blasts for diagnostic purposes (see previous paragraph).

Definition of antigens useful for the detection of minimal residual disease. In the case of first-observation typing, antigens that need to be studied to assess minimal residual disease in follow-up samples should be clearly indicated at the end of the report. This will identify, at the time of diagnosis, those patients in whom the study of minimal residual disease is indicated.[24]


XY HOSPITAL
Flow Cytometry Unit
Cytometric typing of acute leukemia

First name: Xxxxxxxx
Last Name: Yyyyyyyy
Date of birth: mm.dd.yyyy
Case number: 2412 Hosp-code: 78134
Division: Hematology (phone 2241)
Specimen: Bone Marrow
First obs: X Follow up:
Diagnostic suspect: acute leukemia

CD45-FITC
SSC

Panel: CD45, CD5, cCD3, CD2, CD5, CD7, CD56, CD16, CD19, cCD79a, CD22, CD10, CD20, SmIg, cμ, TdT, CD34, CD15, CD33, CD117, CD14, CD15b, MPO. Technique: four colour IF

General features. Bone marrow aspirate characterised by high cellularity (50,000 cells per microliter) and high viability (>99%); several fragments are visible in the specimen.

Results of the gating procedure
- blasts dominate the cell suspension
X blasts clearly detectable among other cells
- blasts undetectable by this procedure

Presence of a blast cell population (orange, 42%) characterised by high expression of CD34, along with middle level expression of CD13, CD33, CD19 and CD56. Erythropoiesis almost absent (0.5%). The maturing granuloid population (blue, 41%) when analysed by CD11b e CD16 shows abnormal differentiation with defective display of CD16. Presence of normal residual lymphocytes (pink, 16%). Acute myeloid leukemia CD34+++CD19+. The results suggest to complete the diagnosis by searching for t(8;21) translocation and AML1/ETO transcript. MRD will be analysed by studying the simultaneous expression of CD34, CD19 and CD56.

Date of typing Signature

Figure 1. An example of a flow cytometry report regarding the characterisation of a patient with acute myeloid leukemia. IF: immunofluorescence; cCD3, cCD79a, cm: cytoplasmic antigens; SmIg: surface membrane immunoglobulin; SSC: side scatter; MRD: minimal residual disease.

An example of a final report

Figure 1 shows an example of a clinical report. The report refers to a case of AML with t(8;21) translocation.[25] In preparing this report, we followed all the 13 points of the checklist described in this paper.

The report is subdivided into six sections. The first reports demographic data, along with the name of the Division that sent the sample, the type of specimen, the first observation/follow-up assessment and the diagnostic suspicion. The second section shows a dot-plot, chosen from among the most relevant findings to allow a better understanding of the final diagnosis. The third section reports the list of antigens with the indication, where applicable, of intracellular staining and the technique used. The next section includes general features of the specimen (cellularity, viability, presence of fragments). The fifth section reports the results of the gating procedures and refers to the presence/absence of blast cells. The last quadrant is the core of the report, and gives the *pathologist-like* detailed description of leukemic cells, as well as of normal surrounding cells. It also contains references to the strategy to be used to study minimal residual disease during the follow-up.

The example refers to a first observation immunodiagnosis. In the case of a follow-up analysis, the same checklist should be adhered to. Differences will be confined to the last section, in which refractoriness, overt relapse, remission status or presence of minimal resid-

ual disease will be documented. It is conceivable that during the follow-up, all bone marrow aspirates taken for disease status assessment will be sent to the flow cytometry laboratory. Early response to therapy is now considered one of the most relevant prognostic factors in acute leukemia. Thus, the report on the flow cytometric characterization of the first BM sample obtained after induction therapy will play a key role, providing important information for risk assessment of patients.[26]

LDV, BB, FL, CO, GP, GS and GB were responsible for analysis of literature data and of their own flow cytometry archives. All the authors were equally responsible for the preparation of the recommendations. LDV was responsible for writing the manuscript. All the authors were responsible for the final revision of the paper. The authors reported no potential conflicts of interest.

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