Consensus on minimal requirements for flow cytometry immunophenotyping of hematological malignancies in Romania

Adriana Dumitrescu[1], Mihaela Zlei[2], Mihaela Baica[3], Horia Bumbea[4], Doina Barbu[5], Grațiela Tardei[6]

1. Fundeni Clinical Hospital, Bucharest
2. "St. Spiridon" Clinical Emergency County Hospital, Iași
3."Louis Turcanu" Pediatric Hospital, Timișoara
4. Emergency University Hospital Bucharest
5. Colțea Hospital, Bucharest
6. „Victor Babeș" Hospital of Infectious and Tropical Diseases, Bucharest

Summary

On the occasion of the 6-th National Congress of Cytometry, organized by the Romanian Association of Cytometry in 2010, the Working Group for Standardization in Cytometry was established. The objective of the first meeting of the group was to establish methodological recommendations for Flow cytometry laboratories from Romania with hematological profile, where rutine immunophenotypic analyses are performed for the diagnosis of hematolymphoid neoplasias. Here there will be discussed the recommendations of several international scientific organizations addopted by the group, with focus on: medical indications, request forms for immunophenotyping, pre-analytical precautions, sample preparation, cell staining with monoclonal antibodies, panel formulation, data acquisition, data interpretation, report of results, quality control. On a long term, these recommendations are intended to set a foundation for unitary, standardized protocols to be used nationwide, in order to increase the efficiency of this type of investigation and, consequently, the efficiency of the therapeutical decision.

Key words (6): flow cytometry, differential diagnosis, hematology, leukemia, lymphoma, standardization.

Introduction

According to WHO classification of hematological malignancies introduced since 2001 as an international standard, the main leukemia/ lymphoma subtypes to be taken into consideration are rather based on clinico-biological entities than on histopathological subtypes (1-6).

As a consequence, the diagnosis of such diseases requires combined information provided by histopathology and/ or cytopathology, immuno-phenotypic analysis, molecular/ genetic findings as well as by the clinical behavior.

Flow cytometry, a highly complex technology, remains the preferred method for the immuno-phenotyping of malignant cells as an indispensable diagnostic tool in oncologic hematology.

Standardization efforts of several scientific organizations have led to the development of some recommendations regarding the terminology, indications, methodology and interpretation of the immunophenotypic analysis of hematological malignancies (7-14).

Despite all these efforts, additional work is required to define in a unitary manner the specific methodology necessary for obtaining clinically relevant information, at the lowest possible cost and with no negative impact on quality (15-17).

On the occasion of the 6-th National Congress of Cytometry, organized by the Romanian Association of Cytometry in 2010, the Working Group for Standardization in Cytometry was established.

The first meeting of this group took place on July the 2-nd, 2010 (List of participants, in alphabetical order: Mihaela Baica, from "Louis Turcanu" Pediatric Hospital, Timisoara, Doina Barbu, from Coltea Hospital, Bucharest, Horia Bumbea, from Emergency University Hospital Bucharest, Adriana Dumitrescu, from Fundeni Clinical Hospital, Bucharest, Gratiela Tardei, from „Victor Babes" Hospital of Infectious and Tropical Diseases, Bucharest, Mihaela Zlei, from "St. Spiridon" Clinical Emergency County Hospital, Iasi). The objective of the meeting was to develop methodological recommendations for Flow cytometry laboratories from Romania with hematological profile, where routine immunophenotypic analyses are performed for the diagnosis of hematolymphoid neoplasias. On a long term, these recommendations are intended to set a foundation for unitary, standardized protocols to be used nationwide, in order to increase the efficiency of this type of investigation and, consequently, the efficiency of the therapeutical decision.

1. Clinical indications

In order to ensure an optimal balance between cost and efficiency in this field it is strongly recommended that this investigation be requested only when its relevance is guaranteed by a judicious selection of

samples.

Flow cytometric immunophenotyping should be recommended by the physician only after the clinical and paraclinical examination of the patient and, cyto-morphological evaluation of peripheral blood/bone marrow aspirate specimens becomes suggestive for the diagnosis of leukemia/ lymphoma.

Flow cytometric immunophenotyping should be recommended for the differential diagnosis between acute leukemia (AL) and myelodisplasic syndrome (MDS), for AL classification, for diagnosis and classification of chronic lymphoproliferative disorders (CLPD). Also, immunophenotypic analysis might be useful to evaluate the prognosis in AL and CLPD patients. Flow cytometry is also useful in monitoring of AL, CLPD, MDS and may be recommended if there are sufficient financial and human resources.

Monitoring comprises changes in the progression of the disease, evaluation of response to treatment and detection of minimal residual disease.

Immunophenotyping is not indicated in myeloproliferative disorders (MPD) as long as the disease remains chronic or stable, but might prove useful for monitoring the course of the disease.

2. Minimum Required Information to be Submitted With a Patient Sample

A patient sample should be submitted with a Flow Cytometry Test Request Form to assist the laboratory in proper sample processing and interpretation of results and to allow their comparability in multicenter national or regional studies. In the Flow Cytometry Test Request

Form the following information should be mentioned:

- patient demographics
- patient's date of birth/ age (the numerical code from Romanian ID cards contains information for both the gender and the date of birth)
- sample type
- the suggestion for an appropriate diagnosis/ clinical diagnosis and any additional information (previous history when relevant)
- date and time of collection
- the test name
- signs or symptoms leading to test request (check all that apply).

Lymphadenopathy (include most recent cytology report if available)
Splenomegaly
Hepatomegaly
Lymphocytosis (lymphocyte number/ the most recent blood cell count/ cytology report if available)

Other peripheral blood abnormality (the most recent blood cell count/ cytology report if available)

Bone marrow abnormality (include most recent cytology report if available)

Mass (location/ the most recent cytology report if available)

Effusion/fluid containing suspicious cells (pleural/ peritoneal/ cerebro-spinal fluid/ other)

Other signs and/or additional history or concurrent conditions including any relevant treatment.

3. Preanalytical precautions (specimen collection and handling)

The Romanian Working Group for Standardization in Cytometry discussed and adopted as a main reference the recommendations of ELN – Work Package 10, Diagnosis (18).

Briefly, in case of AL, bone marrow is preferred, but peripheral blood samples with large blast counts (>80% or >30 G/L) can also be used. Peripheral blood can be used in those cases where it is impossible to obtain an adequate bone marrow sample, i.e. in case of marrow fibrosis. To avoid dilution of the sample of bone marrow blood no more than 5 mL should be collected in one spot. For CLPD diagnosis with lymphocytosis, peripheral blood is usually tested; however, bone marrow, lymph node suspensions (fine needle aspiration for primary screening to avoid unnecessary biopsies) can be analyzed. The preferred choice of anticoagulant is the tripotassium salt of ethylendiamine tetraacetic acid (EDTA K3), whereas heparin should be used only as a second choice because it may alter cell morphology.

The sample should be kept at constant room temperature (22–25°C) until processing, which should be performed within 72 h after collection. However, it is strongly recommended that the samples be processed within the first 24 h.

Clotted, haemolyzed, refrigerated or frozen specimens are not acceptable. However, the rare nature of the specimens submitted for analysis dictates that every attempt should be made to derive useful information from any specimen submitted for analysis. The poor quality of the sample (clotted, hemolysis, lack of granules, hypocellularity) will be mentioned in the final report.

4. Technical aspects

4.1. Preparation of Single Cell Suspensions

Specimens for flow cytometric immunophenotyping must be processed into single cell suspensions. For most hematological specimens (blood, bone marrow aspirates) this is rarely an issue. One exception occurs when a partially clotted specimen is accepted for analysis. In this instance, the clot or clots should be disrupted to release any viable cells.

For tissue biopsies, including fine-needle aspirates and bone marrow biopsies, obtaining single cell suspensions with a maximum yield and preserved cell integrity is a more complex issue. Most lymphoid tissues are easily dissociated by mild mechanical means

(scalpel, forceps, needle and syringe, wire mesh screens, or automated devices) performed in an isotonic buffer or nutrient medium.

Although all immunophenotyping laboratories should have their own validated standard operation procedures (SOP), there are some consensual remarks about sample preparation. The cell concentration should be adjusted to less than 10 million/ mL (500000-1000000 cells/tube). If the multiparametric analysis involves the use of more than four colors simultaneously, the sample volume must be at least 100 ml/ tube. It is recommended that immunophenotypic analysis be performed according to whole sample lysis procedure, avoiding density gradient separation, as follows:

- to detect cell surface antigens: stain, lyse, wash and fixe the samples;
- to detect cytoplasmic antigen, use commercial fix and perm reagents before staining.
- to detect immunoglobulin light or heavy chains, first remove soluble immunoglobulins by washing the cells, then continue with staining, lysing, washing and fixing the samples.

Use commercial reagents for erythrocytes lysis.

Lyse and wash vs lyse no wash protocols are increasingly preferred (18).

Fixation with paraformaldehyde 0.1 to 0.5% in Phosphate Buffered Saline (PBS) after surface staining is not mandatory if the flow cytometry acquisition is carried out immediately after sample preparation, but is recommended. This procedure will preserve the fluorescence over several days if fixed samples are kept in the dark, at 2-8°C, and will also neutralize any virus present in the sample. However, as light scatter and fluorescence intensity of stained cells might change during refrigeration, even after fixation, it is advisable to avoid postponing flow cytometry acquisition of processed samples more than 24h. Refrigerated samples should be equilibrated for 30 minutes to room temperature before acquisition.

4.2. Staining with Antibodies

The number of antibodies and fluorochromes that can be combined will obviously be limited by the capability of each instrument. Each laboratory should decide on how to combine the different fluorescent antibodies recommended in the minimum panels (19).

Regarding the choices of fluorochromes, it was recommended that in each antibody mixture, phycoerythrin (PE) or allophycocyanin (APC) should be chosen as the label for that antibody directed to the antigen with the weakest expression, whereas antibodies conjugated to other fluorochromes should be used for the detection of antigens with higher density. There is large agreement today that the use of fluorochrome-conjugated monoclonal antibodies is superior to unconjugated antibodies in double-antibody staining procedures (19). It is recommended to use antibodies in excess amounts, determining the optimum concentration by preliminary titration.

4.3. Antibody panels

It is recommended that the immunophenotypic analysis be performed in a sequential manner, both for a better characterization as well as for economical reasons, when diagnostic, classification and follow-up of leukemias & lymphomas are required.

Panels should include combinations of backbone markers (for gating purpose) and characterization markers for selecting and defining the cell populations of interest, including detection of phenotypic aberrations, needed for appropriate follow-up of minimal residual disease. The reagents recommended are those that will provide immunophenotypic valuable in making clinical decisions. Any attempt to reduce the number of monoclonal antibodies used must be cautious, in order to avoid decreasing the clinical value of the assays (17).

The Romanian Working Group for Standardization in Cytometry adopted as a main reference the recommendations of WP10 – ELN regarding consensual European immunophenotyping panels for leukaemia (20).

According to these recommendations a mandatory panel for diagnosis of AL patients should include 27 markers. For quick orientation or for paucicellular samples: cCD3, MPO, cCD79a, TdT, CD7, CD2, CD10, CD19, CD22 (s or c), sIg, CD13, CD33, CD34, CD45 for gating purposes; for sublineage classification and definition of clinical entities: HLA-DR, CD1a, CD4, CD5, CD8, CD3 (m), IgM (c), CD14, CD117, CD56, CD65, CD41 (or CD61), CD235a (or CD36). Other markers (>20) are useful for better identifications and classification of AL entities: MPO/LF (lactoferrin), LZ (lysozyme), kappa/ lambda, CD11b, CD11c, CD15, CD16, CD35/36, CD58, CD64, CD68, CD71,CD86, CD99, CD123, TCR chains. A mandatory panel for diagnosis of CLPD includes 21 markers: CD19, CD3, CD56 (gating markers), CD5, CD20, CD22, CD23, CD103, FMC7, CD10, kappa, lambda, Ig, CD25, CD79b, CD38 (B- cell oriented panel gated on CD19), CD2, CD3, CD4, CD5, CD8, CD7 (T- cell oriented panel gated on CD3 or other T-lineage marker). An additional panel should include other useful markers (<15): for B lineage: CD27, CD123, CD138, DR, CD24, CD43, Ig (G, A, M, D), CD81, Bcl2, Zap70; for T lineage: TCRs, CD30, CD10; for NK cells: CD57, CD16, CD94, perforin, granzyme B. In the particular case of multiple myeloma a mandatory panel should include: CD38, CD19, CD138, CD56, CD45 and cytoplasmic light chains.

4.4. Data acquisition and storage

Once daily instrument settings have been verified, acquisition of processed patient samples can proceed. Listmode data should be collected on each sample that is acquired with appropriate demographic and sample information saved. It is recommended to collect ungated data on specimens in which the nature of the abnormal cell population is unknown so that no important population would be missed on subsequent analysis of the listmode data. The minimum number of events of interest to be acquired and available for analysis, particularly when dealing with the quantification of minimal residual disease, is recommended to be 100, for reliable statistics of a given cell population (reproducibility, coefficient of variation, and count). This goal, in many instances, requires acquisition of several hundred thousands to millions of total events. A minimum of 20 clustered events is required to define a population in paucicellular samples like cerebrospinal fluid.

4.5. Data Analysis

Data analysis has two major objectives: 1. discrimination of abnormal cell populations (potentially neoplastic) from normal; 2. identify the antigenic profile of neoplastic cells.

For the discrimination of abnormal cells from residual normal cells and for an efficient discrimination between the various cell lineages, the use of CD45/ SSC gating procedure is highly recommended.

Characterization of the antigenic profile of neoplastic cells should include the presence or absence of each cell surface marker, as well as the intensity of its expression. It is recommended that interpretation of results be based on classification systems developed by EGIL and/or WHO (1-6, 20, 21) allowing assignment of lineage, stage of maturation, identification of aberrant phenotype, and identification of specific pattern for some entities. Immunophenotypic data obtained should be correlated with other laboratory data (morphology, genetics, molecular biology) and interpreted in clinical context.

5. Report of Results

According to suggestions made by Del Vecchio in 2004 (22), the report should be comprehensive and interpretative.

The report should include the following sections:

- Demographical data (gender, and age; the numerical code from Romanian ID cards contains information for both the gender and the date of birth).

- Clinical information. Suspected diagnosis as established by the referring clinician and potentially useful clinico-biological information associated with the patient; name of clinician, the hospital and ward who sent the samples.

- Date and hour of sample collection

- Type and quality of the sample. A brief description of the type and analytical quality of the sample (hemolysis, visual clots, or sample deterioration) should be mentioned.

- Antibody panels. The report should include the list of the antibody combinations, including fluorochrome, used in the particular reported case.

- Cytometer type, software, the number of events acquired, date of staining and acquisition

- Results. A succinct, although comprehensive, description of the neoplastic cells should include light scatter properties as well as the presence or absence of each of the tested antigens (positive or negative). Reporting the percentages of cells expressing each one of the antigens is no longer recommended, as it has a limited relevance (8). Information regarding the intensity, modality, and coexpression of several antigens should be provided as well. The adjectives dim or bright should be used as appropriate, when the relative intensity of expression of a given antigen differs from normal. When pertinent, the multimodal (most commonly bimodal) expression of a certain antigen should also be stated.

- Interpretation. The concise immunophenotypic diagnosis is to be given, when applicable. The interpretation of the immunophenotype should always be stated, preceeded by the word compatible or consistent with, e.g., "Compatible with B-cell line acute lymphoblastic leukemia" (in a case where CD19 and CD79a were present in the absence of highly specific markers of other lineages). In institutions where antibodies in addition to the minimum panel are used, additional information may also be included in the interpretation, e.g., "Consistent with common acute B lymphoblastic leukemia" (if CD10 was also present in the absence of cytoplasmic μ and surface k and l light chains). When all the clinical information including morphology is available and there is no doubt concerning the diagnosis, the word compatible may be omitted (8).

- Cytogram(s). The inclusion of one or more representative cytograms of the core findings remains optional.

6. Quality control

6.1. Sample Process and Analysis Controls

All specimens submitted for analysis of hematological malignancy contain residual normal hematopoietic cells. Therefore, panels should be configured such that the normal cells present provide distinct positive and negative controls for each antibody combination in use. Thus, there is strong consensus that there is little or no need for a normal donor process control to be run with each batch of patient specimens analyzed on the flow cytometer (8, 23).

Likewise, each antibody panel combination should

be designed to provide clear thresholds for the interpretation of "positivity" of each marker, based on negative cell populations present in the same sample. Thus, there is strong consensus that specific isotype controls provide no useful additional information beyond unstained cells alone, or negative cells in selected antibody combinations (8).

6.2. Instrument Quality Control and Quality Assurance

In order to achieve an optimum interlaboratory comparability of fluorescence intensity measurements, a rigorous and standardized approach to instrument quality control and quality assurance is required. Instrument quality control programs generally consist of instrument setup (i. e. to establish the initial performance characteristics for use in analyzing stained samples) and instrument performance monitoring (i. e. to validate that daily instrument performance is consistent with the measurement criteria established at instrument setup) (8).

If any of instrument variables (photomultiplier tube voltage, gain laser power, optical filters themselves) are changed, instrument setup must be repeated.

The minimal instrument setup criteria presented here ensures that the flow cytometer is performing in a manner sensitive enough to allow for the discrimination of unstained lymphoid cells from instrument noise. A flow cytometer set up in this mode will be readily capable of discriminating weakly expressed antigens commonly encountered in this application.

In addition, in order to remove the unwanted contribution of fluorescent light from a dye with an overlapping emission spectrum, correction protocols are required, known as compensation protocols. Fluorescence compensation is accomplished either by electronic hardware subtraction from the detector of any unwanted fluorescence signal contributed by the other overlapping fluorochromes used in the analysis, or by applying mathematical algorithms to the uncompensated listmode data using software designed specifically for this purpose (8).

6.3. Reference materials for daily instrument QC

Use of cells as reference particles. Cells stained with mutually exclusive antibodies in the fluorochrome combinations of interest make good reference materials for establishing and verifying appropriate color compensation settings. Color compensation should be adjusted after all other instrument settings and filter combinations are finalized. Use of particles as reference materials. Another source of reference material is plastic microbead particles with multiple levels of fluorescence intensity ranging from negative to very bright and labeled with the same specific fluorochromes to be used in the multicolor analysis of specimens.

Conclusions

The current recommendations are intended to set a foundation for unitary, standardized protocols to be used nationwide as well as to increase the clinical utility of this investigation while keeping the cost of immunophenotyping as low as possible. However, we are aware that the additions or amendments will become necessary in the future because, as it has been stated by Paietta, "although immunophenotyping by flow cytometry has become a routine approach to the diagnosis of hematological malignancies, it is a discipline that is still in development" (24).

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Address for correspondence
Adriana Dumitrescu
Fundeni Clinical Hospital
258 Fundeni Str.
022328 Bucharest
Phone/Fax +40 21 313 1015
amdumitrescu@yahoo.com