FLOW CYTOMETRY'S CONTRIBUTION TO THE DIAGNOSIS OF THE MALIGNANT HAEMOPATHIES

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Abstract: Flow cytometry represents an indispensable examination for the malignant haemopathies diagnosis. The existence of an increasing number of fluorochromes in combination with the use of different lasers greatly enhances the versatility of flow cytometry. In myeloid acute leukaemias, CD33, MPO, and CD13 are the most frequently expressed antigens, followed by CD65s and CD15. The most frequently expressed antigens in B-lineage of acute lymphoblastic leukaemias are cyCD79a and HLA-DR, and in T-lineage of the same disease are cyCD3 and CD7. For the diagnosis of chronic lymphocytic leukaemia, the following antigens are useful: CD5, CD19, CD20, CD23, HLA-DR. Many markers are associated with an unfavourable prognosis in certain neoplasms, and some antibodies against lymphocytic and myelocytic antigens have been used effectively for the treatment of lymphoma and leukaemia.
Keywords: flow cytometry, leukaemia, lymphoma, antigens, antibodies
Rezumat: Flowcytometria constituie o examinare indispensabilă pentru diagnosticul hemopatiilor maligne. Existenţa unui număr crescând de fluorocromi în combinaţie cu folosirea de lasere variate a crescut mult adaptabilitatea flowcytometriei. În leucemiile acute mieloide CD33, MPO şi CD13 sunt antigenele cel mai frecvent exprimate, urmate de CD65s şi CD15. Cele mai frecvent exprimate antigene în leucemia acută limfoblastică tip B sunt cyCD79a şi HLA-DR, iar în tipul T al aceleiaşi afecţiuni - cyCD3 şi CD7. Pentru diagnosticul de leucemie limfatică cronică sunt utile următoarele antigene: CD5, CD19, CD20, CD23, HLA-DR. Mulţi markeri sunt asociaţi cu un prognostic nefavorabil în anumite neoplasme, iar anumiţi anticorpi faţă de antigene limfoide şi mieloide au fost efectiv folosiţi pentru tratamentul limfoamelor şi leucemiilor.
Cuvinte cheie: flowcytometrie, leucemie, limfom, antigene, anticorpi

There have been made a lot of progress in the filed of haematology lately, so that most of the times, in order to establish the diagnosis of a malignant hemopathy, the objective examination and that of the myelogramme in optical microscopy are not sufficient. Flow cytometry is also needed in this case, as well as the cytogenetic and molecular biology examination; initially and in dynamics, the following are not exceptional, as well: enzymatic cytochemistry, hybridization through fluorescence in situ and the radiological examinations, including the computerized tomography or positron emission tomography; moreover, the even more complex haematological pathology and the severe complications that may occur, make the physicians resort to any kind of laboratory analyses in the filed of microbiology, virology (including those that involve the chain polymerization reaction), parasitology, blood coagulation tests, fibrinolysis and others. It is a true art to combine the diagnosis methods in order to reach the most accurate diagnosis as possible.

The old classification of leukaemias and lymphomas was strictly based on morphology, while the Euro-American Classification of the World Health Organization requires a morphological, immunophenotypic and molecular-cytogenetic correlation in order to put a final diagnosis. (1) Due to the recent progress in the treatment of neoplasms, the sub-classification of the malignant hemopathies is important in order to accurately choose the therapeutic agents indicated for the different hemopathies. It is also essential to mention the contribution of the immunophenotypation and that of karyotype in formulating the prognostic. (2)

In the last 10-15 years, large improvements have been made regarding the hardware and software of flow cytometres, but the existence of an increased number of flourochromes in combination with the use of different lasers brought about the increase of flow cytometry adaptability. (3) For example, a flow cytometre with 3 colours may detect the coloration for lambda and kappa chains in one single tube of cellular suspension. A flow cytometre with 5 colours may identify the lambda, kappa chains, CD19 and CD5, and one with 6 colours may prove the existence of lambda, kappa, CD19, CD5 and CD23 in one single tube. Thus, a little blood sample is now efficient for a large panel, and a large panel of antibodies marked with a fluorescent marker is able to characterize the tumoral cell population more accurately regarding a certain diagnosis. (2)

A group of researchers from Napoli made a recommendation regarding the issuance of the flowcytometric report, comprised in the guide of the

Italian Society for Flow cytometry. It is indicated that the report should contain: 1. patient's identification data; 2. identification data of the hospital and of the department which sent the biological sample; 3. blood type (spinal cord aspirate, peripheral blood, other biological fluids); 4. moment of observation (as a first diagnosis and in supervision); 5. diagnosis formulated by the physician; 6. list of antigens and the type of the accomplished immunofluorescence; 7. the absolute number of the sample cells; 8. quality of the sample in terms of viability; 9. general description of the procedure; 10. immunophenotype of the blast population; 11. description of the cells surrounding the blasts; 12. definition of a panel of antigens (when applicable) for the detection of a minimum residual disease. (4)

Acute leukaemia

The diagnosis of the types of acute leukemia (AL) may be accomplished immunologically, by using a 33-marker panel and without consideration of the morphocytochemical parameters of blast cells. But such an approach complicates and prolongs the examination of patients with AL. Moreover, morphocytochemical data more exactly define the stages of blast cell differentiation than the immunological phenotype does. Their preparation methods are simple and cost-effective. Only M0, M6, and M7 forms of leukemia require compulsory blast cell phenotyping, particularly in the differential diagnosis of acute myeloid leukemia and acute lymphoblastic leukemia. Search for new markers of leukemia cells, including lesions at the chromosomal or molecular levels, is under way. Some of them are only of theoretical value while other markers have been already used by hematologists to diagnose leukemia. (5)

Antigenic expression is considered positive if at least 20% of the cells of a certain type react with a certain antibody. Reactivity of blasts with myeloperoxidasis (MPO) brought about by the combined colouration for myeloperoxidasis and lactopherin (LF) is considered positive if at least 10% of the mononuclear cells are MPO+/LF-. (6, 7)

The samples taken from the patients suffering from leukaemia are initially tested with a primary panel of antibodies and, if necessary, they will be characterized with a secondary panel. If a spinal aspirate is too poor in cells in order that the entire panel of antibodies should be tested, the antibodies will be selected according to the morphologic phenotype or, according to the degree of the line specificity. The primary panel of antibodies marked with fluorescent colour, indicated for the diagnosis of acute leukaemia will comprise: cytoplasmatic MPO, cytoplasmatic LF, CD13, CD33, CD14, CD15, CD65s, blood type antigen H, CD61, CD79a cytoplasmatic, D22 cytoplasmatic, CD10, CD19, CD3 cytoplasmatic, CD2, CD7, CD56, CD34, HLA-DR, CD117, TdT. The secondary panel will comprise: cytoplasmatic and serum IgM, CD20, CD24, CD22, CD23, kappa, lambda, CD1a, serum CD3, CD4, CD8, CD5. (6)

In this study, the most frequent myeloid antigens were: CD33, MPO and CD13, followed by CD65s and CD15. 22,4% of the myeloid acute leukaemias co-expressed lymphoid antigens. Five acute myeloid leukaemias expressed two lymphoid antigens at the same time (CD7 and CD56, CD2 and CD7, CD2 and CD56, CD2 and cyCD79a). All samples of acute B lymphoblastic leukaemia expressed cyCD79a. CD19 was expressed in 47 out of 48 of acute B lymphoblastic leukaemia cases. 9% of acute B lymphoblastic leukaemia co-expressed myeloid antigens. There were 4 biphenotypic acute leukaemias in this study. Myeloid-type blasts expressed MPO. All leukaemias expressed cyCD3, MPO, CD7 and CD34; 3 leukaemias expressed CD2. The expression of the myeloid surface markers - CD13, CD33, CD15 and CD65s were randomized among the four cases of leukaemia; one case expressed cyCD79a. (6) There was another study that reported a case of biphenotypic acute leukaemia, where the spinal and peripheral blasts expressed: CD19, CD79 alpha intense, CD22 and TdT as lymphoid markers, CD13, CD117, CD15 as myeloid markers and CD34, HLA-DR as markers of the stem cells. Myeloid antigen CD33 was expressed in 50% of the blast population. There were no differences in the immonophenotypic profile between the two populations of blasts morphologically identified and where the correlation for MPO was negative. The patient made the induction therapy for acute myeloid leukaemia, followed by the autografting of peripheral stem cells, but the recurrence occurred 6 months after. (8)

A score system was created regarding the most predictive phenotype of the acute myeloid leukaemia – M0 and acute lymphoblastic leukaemia. The following observations were taken into consideration: CD13 and CD33 were distinctive among the myeloid associated antigens; CD117 was detected almost exclusively in the acute myeloid leukaemia-M0; lymphoid-associated antigens cyCD79a, cyCD3, CD10 and CD2 were exclusively expressed by acute lymphoblastic leukaemia. (6)

The most significant markers, immunologically distinctive between acute myeloid leukaemia-M0 and acute lymphoblastic leukaemia were: CD13, CD33 and CD117, typical for M0 and cyCD79a, cyCD3, CD10 and CD2, that are typical either for the B cell line, or for the T cell line. CD15 and CD65s were expressed in the precocious precursors to the acute lymphoblastic leukaemia rather than in M0 and this is the reason for having not been chosen for the line differentiation. With the help of the proposed score system, all acute myeloid leukaemia –M0 evaluated cases could not be differentiated from acute lymphoblastic leukaemia. (6, 9)

In the adults with acute T-lineage lymphoblastic leukaemia (T-ALL), CD5 expression was observed more frequently than CD2. CD5 is almost never expressed in acute myeloid acute leukaemia- M0 (AML-M0). Adding CD5 as an alternative to the CD2 antigen may increase the sensitivity of the proposed score system in order to detect T-lineage lymphoblastic leukaemia.

Taking into account the experience of the cited authors, it is suggested that together with the

morphological and cytochemical examination, a panel of monoclonal antibodies as against MPO, cyCD3, cyCD79a, CD13, CD33, CD10, CD19, CD2 and CD117 may be cost-efficient and may represent a high predictive screening for the lineage differentiation of the acute leukaemias. (6)

Chronic lymphatic leukaemia

Today, chronic lymphatic leukaemia (CLL) is easy to be diagnosticated through flow cytometry, that may detect the co-expression of CD5 and CD19, as well as that of CD20 and CD23 (11) and HLA-DR. (12) CD19, CD20 and CD23 are antigens typical for the B cell. On the contrary, CD5, previously believed that is a T cells-specific antigen, is considered today a characteristic marker for the CLL diagnosis, if it is co-expressed with the antigens of the B cells, while only a small sub-set of normal antigens co-express the B-cell surface antigens and CD5. (11) The study of the flow cytometric markers with B cells in the patients who started the chemotherapy concluded that they had a slightly decreased level of expression, almost insignificant, in the case of CD19, CD20, CD23. CD5 registered higher values than before the therapy. The values of the fluorescence intensity expressed in molecular equivalents of the soluble fluorescence for CD19 and CD23 was significantly more reduced than in the untreated patients. (12)

Study of lymphomas

Immunohistochemistry (IHC) is an important method for the hematopathological diagnosis of lymphomas. The initial panel of IHC includes the B and T-anti-lymphocytes antibodies (CD20), (CD3); as against the easy lambda and kappa channels (if numerous plasmocytes are present); as against the CD45, CD15 and CD30, if large dysplastic cells could be detected in optical microscopy. Based on morphology, if there are alternations with the B lymphocytes, it is suggested to use antibodies against CD5, CD10, CD23, CD43, BCL-2, BCL-6, that are useful for the subsequent characterization of the process. If there are expansions of the interfolicular areas and a possible lymphoma with T-cells is studied, antibodies against CD2, CD4, CD5, CD7 and CD8 will be used, that will identify the distribution of the sub-sets. CD20 is the most used pan-B cellular marker and it is expressed from the B naïve cell up to the final stage of the development of the B cell, before the plasmocytary differentiation. CLL may be slightly positive or occasionally negative for CD20. This pattern of coloration is similar with the flow cytometry observations, where CD20 is frequently low expressed as against the normal B lymphocytes or against other B cells lymphocytes. If immature, abnormal blasts are present, or if the patient received rituximab (anti-CD20 antibodies), other antibodies must also be included in order to detect the B cells differentiation, such as: CD79a or Pax-5 (B-cell specific activator protein). (13) The antigens that require extra study, in case of lymphocyte proliferation with large cells are: CD15, CD30 and CD45RB. CD30 is an activator antigen, jointly expressed in the large cells, at the periphery of the B-cell follicles and in interfolicular

areas; in the reactive processes, such as: infectious mononucleosis, numerous, large and dysplastic cells, CD+30 may also exist. The anaplastic lymphomas kinase (ALK) and the protein kinase of CD15 are useful for the evaluation of the large dysplastic cells, CD30+. The lack of the CD15 expression does not exclude the classic Hodking's lymphoma. (13, 14)

Neoplasms with small B cells: neoplasms expressing CD5 but with no expression of CD10, include the lymphoma with lymphomatous polyposis and CLL. A reduced number of CD+5marginal area MALT lymphomas were also reported in flow cytometry. A small number of follicular lymphomas (5-30%) and of CLL (below 1% in flow cytometry) are CD5- and CD10-. Almost all neoplasias with B small cells expressed BCL-2 – antiapoptotic molecule. (13)

Explanations regarding the T lymphocytes markers

CD3 is the most frequently used pan-T marker. It is normally expressed starting with the second stage of the timic differentiation and subsequently. It may be lost in certain neoplasms with T cells, particularly in the lymphoma with large anaplastic cells. Flowcytometric detection of surface cell CD3 is a definitive proof of the T lineage, as it is the expression of the $\alpha\beta$ protein of the T cells. The normal relation between CD4/CD8 is 1,5-3:1. The majority of the reactive processes evolve with the predominance of the T CD4+cells. In the absence of the HIV infection or of other virus infections, a neoplazic process with T or NK cells must be taken into consideration if a marked predominance of the T CD+8 lymphocytes is present, especially in extranodal sites.

CD5 is a pan-T antigen and is not expressed in the NK cells. Still, CD5 is not lineage-specific. CD5 is presented only in a very small subset of normal B cells, but most importantly is the fact that CD5 is expressed in CLL and more reduced, in a small sub-set of B cells neoplasms. If CD5 is expressed in B areas, it is necessary to determine the type of neoplasm with B small cells; if its expression is lost in the areas with T cells, a possible lymphoma with T or NK cells must be studied. (13, 15)

Cyclynes study

In a study made on a batch of 80 patients with chronic lymphoproliferative actions with CD5 B positive cells, it was noticed that D1 and D2 cyclynes were intensely expressed in the lymphomatoid polyposis; regarding the prolymphocytic leukemia, in 4 out of 5 patients, D1 cyclyne was strongly expressed while D2 cyclyne was weakly expressed. The fluorochrome coloration for both cyclones was weak in the CLL cases. The coloration for the D3 cyclyne was weak in all three neoplasias. The levels of D1 cyclyne were significantly higher in ZAP-70 positive cases, while no association between ZAP-70 and D2 cycylne was found. (16)

D1 cyclyne is aberrantly expressed in a sub-set of myelomatous cells. The intense coloration for D1 cyclyne was associated with the increased number of plasmocytes in the diagnosis of myeloma, with the lymphoplasmocytary morphology, with the CD20 expression and with the t translocation (11;14) (q13;q32),

detected through fluorescence hybridization in situ. On the contrary, the weak coloration was associated with hyperdyploidy and gaining the CCND1 locus. The global survival was high in the cases with D1+cyclyne as against those with D1- cyclyne. It results that D1 cyclyne may offer prognostic information in the patients with multiple myeloma. (17)

Sezary syndrome

The recommended criteria for the diagnosis of the Sezary syndrome are: an absolute number of Sezary cells of at least 1000/B5L, proving the immunophenotypic anomalies through the expansion of the CD4+ T cell population that leads to a relation of CD4/CD8 higher than 10, the loss of CD4 and/or CD5 and/or the increase of the number of lymphocytes with the evidence of a T cell clone upon the cytogenetic examination. It is still under debate whether adding the loss of CD7 to some of the CD4+ T cells is pertinent. Regarding the loss of CD26 expression, it was identified in 59,3% of the patients with Sezary syndrome, in 33% of those with mycosis fungoides and in 14,2% of those with benign dermatitis. The loss of the antigen of the T cells was significantly higher in those with Sezary syndrome as against those with mycosis fungoides. (18)

New antibodies

In the last years, it has been discovered that new antibodies are being produced. Some of them are particularly useful. For example, CD79 is an antibody that may detect the antigen of the B cells in the entire spectre of the development of the B cells: from the B precursory cell to plasmocyte. These markers may be proved in almost all neoplasms with B cells, with a few exceptions, such as: chronic lymphatic leukaemia. CD138 is a strong marker for the identification of plasmocytes. CD117 compensates for the weak expression of CD34 at blasts level. It may detect the blasts in all acute myeloid leukaemia (AML), including AML3, where CD34 is negative. Moreover, it is negative in lymphoblasts, so that it may help in differentiating the lymphoid leukaemias from those myeloid. On the contrary, CD34 is positive both in AML and in ALL. A relatively new antibody - clusterin – may help in the common ALL diagnoses and may help in the differentiation of the systemic form from that of primary cutaneous. Regarding the latter, there is no clusterin in the tumoral cells in the same concentration. (2)

Evaluation of the nuclear and cytoplasmatic antigens

In the past, flow cytometry was used especially for the study of surface antigens. Adding the coloration of the nuclear and cytoplasmatic antigens makes easier the differentiation of the various cell lines and identifies the different development stages. Cytoplasmatic MPO identifies the myeloid cells, cytoplasmatic CD3 – precursors of the T cells, cytoplasmatic CD22 – precursors of the B cells and deoxynucleotidyl transferase – precursors of the T and B cells. Cytoplasmatic BCL-2 in combination with CD10 identifies the cells with their origin in the cells of the follicular centres. (2, 19)

Unfavourable prognostic markers routinely used are CD38 and Zap-70 in CLL. The over expression of these markers is associated with the unmutant status of the V_H gene in acute leukaemia. (19) A lot of antibodies, as against the lymphocitary and myeloid antigens, are used in the treatment of leukaemias and of lymphomas. Used in combination with chemotherapeutic agents, they allow the reduction of the cytotoxic effects of chemotherapy. One of the most known monoclonal antibodies is rituximab (anti-CD20), used in the treatment of the B-cell neoplasms. Alemtuzumab (anti-CD52) and BL22 (anti-CD22) are also used in the treatment of B-cell neoplasias, while gentuzumab (anti-CD33) – in the treatment of AML. (2)

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