Quantitative identification of blood cell markers in human hematopoietic malignancies with diagnostic and prognostic significance*
Minireview

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Fluorescence intensity (FI) is the basis for classifying phenotypes by fluorescence-label flow cytometry. FI is of a relative value, but with calibration it can be expressed in stoichiometric units called molecules of equivalent soluble fluorochrome (MESF) that reflect the concentrations of the fluorescent conjugates and the receptors they stain. Flow cytometry allows in addition to the determination of positive cells, to establish even the intensity of fluorescent staining, that can be converted into antigen density. The concept of antigen density appears to improve the efficiency of immune techniques in the monitoring of hematopoietic malignances. Quantitative immunophenotyping is thus suitable for the diagnosis of malignancy, contributes to prognosis and could provide new relevant pathophysiological informations. Quantitative analysis of some markers of leukemic cells could be a good model for the study of antigen modulation caused by chemotherapeutic agens. Standardized reagents and techniques for performing quantitative FI measurements on cytometers are just now emerging into practical use. This latter feature should see the expanding application in both, the basic science and medical applications, as the development of therapeutics increasingly targets specific cell receptors. FI data constitute a very important component of the analysis of cells in hematopoietic malignancy; standardized approach to instrument quality control, interlaboratory comparability of FI measuremant and quality assurance is required.

Key words: fluorescence intensity (FI), molecules of equivalent soluble fluorochrome (MESF), quality control, quantitative flow cytometry, leukemia/lymphoma

Quantitative flow cytometry. The essential aim of flow cytometric quantitation of immunofuorescence intensity is to enumerate the number of certain molecules on cells of a given population. During the past 20 years, variety of methods have been developed to approach this aim, all of which are based on the detection of the molecules (antigens) of interest using monoclonal antibodies (mAb) and the quantification of mAb binding using calibration system. Flow cytometric quantitation of immunofluorescence is one of the techniques for which we envisage an extension in clinical applications, i.e., for the immunophenotyping of leukemias and lymphomas and for functional assays of lymphocytes and platelets [1].

Fluorescence intensity (FI) is the basis for classifying phenotypes by fluorescence-labeled flow cytometry. In the sense of quantitation of cellular antigens on cells, we measure the number of antibody molecules bound (ABC) to the cells using well-characterized reagents and standards from which we can determine the fluorescence of each fluorochrome-conjugated antibody [2].

FI is of a relative value, but with calibration it can be expressed in stoichiometric units called molecules of equivalent soluble fluorochrome (MESF) that reflect the concentrations of the fluorescent conjugates and the receptors they stain. The MESF concept indicates that a sample labeled with fluorochrome has the same fluorescence intensity as an equivalent number of molecules of the fluorochrome free in a solution under the same conditions. This fluorescence unit provided researchers with tool to compare flow cytometry data in a quantitative manner over time and across instruments. Although MESF units have been used internationally over this extended period, a precise definition of the unit had not been presented until recently. The definition of the unit was presented in the contex of fluorescence measurement model that

relates the physical characteristics of the sample and the resulting fluorescence signal [1, 3]. At present it is understood that the MESF value of a sample is defined in terms of a specific fluorochrome, and as such must be so indicated. The binding of the fluorochrome-conjugated antibody to the cell can give rise to a spectral shift that yields a significant variation of the integrated signal [4]. The recent development of a more fundamental basis for the MESF assignment has clarified the interpretation of the measured fluorescence intensity in terms of MESF values [3, 4].

FI calibration establishes the true values for the critical parameters of the fluorescence measurement, a useful feature for quality control. Because fluorescence intensity data constitute a very important component of the analysis of cells in hematopoietic malignancy, there is strong consensus that a more stringent and standardized approach to instrument quality control and quality assurance is required [5].

The two different approaches can be distinguished for how to use a flow cytometer for quantitation of immunofluorescence. The first – calibration of a specific instrument for the quantitative analysis of one or more predefined antigens. Optical test materials for the control of the alignment of the instrument are usually used only in a monthly or similar interval. The second – generic setup of a flow cytometer, which will allow the instrument- and assay-independent use of the cytometer for antigen quantitation. This concept has been introduced by Schwartz et al. [6] and will be useful in an exchangeable multi-instrument environment. A typical application would be a general procedure for the quantitative description of the antigenic profile of leukemic blast cells.

Quality control of the flow cytometer for quantitation of immunofluorescence should include the assessment of instrument parameters that affect the accuracy and precision of data [7]. Two groups of procedures can by distinguished. The first group of procedures is carried out at relatively large intervals by service and includes examination of the efficiency and performance of the laser tube, logarithmic and linear amplifiers and optimization of the optical alignment of flow cytometers. The second group of procedures consist of frequent (e.g. daily) monitoring of instrument setup by the operators in order to identify potential problems. After the appropriate instrument settings have been established, the target channels for the relevant fluorescence parameters are recorded using fluorescent reference beads, and the results of the day are then compared with the accepted tolerance limits [8]. The instrument's performance for quantitative fluorescence measurements is monitored by computing calibration plots for each fluorescence parameter. Comparison of means of fluorescence intensities between populations required analysis of pure representative populations, proper selection of antibodies, invariance in instrument sensitivity from day to day, calibration of intensities and correct statistical analysis [9].

In summary, for quantitation of immunofluorescence by the flow cytometer the following criteria are very important:

- the standards and the unknown samples must be run on the same instrument at the same settings
- the excitation and emission spectra of the standards must match those of the unknown samples
- the environment of the standards and the unknown samples must be the same.

The resulting data can be compared among laboratories across the country and around the world over extended periods of time [4, 9].

Quantitative fluorescence in leukemia and lymphoma. Most fluorescence reported in the literature and in patients data is really semiquantitative. Although evaluation of intensity of antigen expression may be of minimal importance in lymphocyte subset analysis, it is a vital component of analysis for lymphoma and leukemia. Neoplastic cells may be recognized due to a failure to express expected antigens or because they express antigens associated with another lineage. In some cases, they can be recognized only because they express antigens at a density that differs from that on normal cells. This abnormal expression of cell surface antigens is of a diagnostic value for certain hematologic malignancies, allowing appropriate subclassification. Numerous publications support the relevance of FI determination in diagnostic [10, 11, 12, 13, 14], differential diagnosis [15, 16, 17], prognostic evaluation [18, 19], and therapy monitoring [20, 21, 22, 23, 24] of leukemias and lymphomas.

Farahat et al. [24] have shown quantitative differences in the expression of terminal deoxynucleotidyl transferase (TdT), CD10 and CD19 between B-lineage acute lymphoblastic leukemia (ALL) and normal bone marrow lymphoid precursors. Normal bone marrow precursors express strong TdT and weak CD10 and CD19 whereas leukemic B cells express weak TdT and strong CD10 and CD19. The clinical significance of detecting minimal residual disease (MRD) in B-ALL could be evaluated by quantitative flow cytometry. These quantitative studies have shown that samples with values similar to those of leukemic B cells with two or three of above markers were considered positive for MRD, whereas samples with values similar to normal B-cell precursors were defined as immunologically free of disease.

Nakamura et al. [25] evaluated the clinical implications of CD45 expression in ALL. There was found a significant correlation between the expression levels of CD45 and the high-risk patients group. However, the intensity of the CD10, CD19, CD20 and CD34 antigen immunoreactivity did not correlate with the CD45 expression. These results show that the levels of expression of the CD45 antigen on leukemic lymphoblasts are significantly correlated with the clinical features and prognosis of childhood ALL patients. Reduced or even absent expression of CD45 is frequently observed in ALL [11, 26].

Ratei et al. [27] reported that the surface density of some markers displayed by autologous lymphocytes can be used to measure the density of target molecules on blasts from acute leukemia and described the use of normal residual lympho-

cytes in ALL cases as an internal quality control for multi-laboratory cross validation. These „internal biological control particles" were found to be better suited for quality control between laboratories. In some T-cell neoplasms the only abnormality detected is in the levels of cell surface CD3 expression [28] and therefore, measurement of intensity of antigen expression is crucial to classification of these neoplasms.

Pereira et al. [29] in the quantitative analysis of the expression of the several antigens studied could reveal some interesting facts of prognostic impact in acute myeloid leukemia (AML). They detected a negative impact of the expression of CD14 on overall survival. Similar findings have been reported also by other authors trying to categorize the number of positive cells. Dunphy et al. [30] analyzed extensively the expression of several antigens that have been associated with the monocytic lineage such as CD14 and CD15. They could demonstrate that, besides variation in expression of monocytic antigens according to cell maturation, a partial or complete deficiency of one or more antigens is frequently found in AML with a monocytic component as well as in chronic myelomonocytic leukemia (CML). Presence or absence of antigen expression may be associated with resistance to chemotherapy, as has already been described for CD14 [31] and CD33 [32]. Pereira et al. [29] could detect among some patients that density of CD45 was an important independent factor of a shorter survival. This parameter showed a positive correlation with expression of CD33, CD15 and CD14, indicating a higher degree of maturity of the blasts. They concluded that a quantitative analysis of the commonly expressed antigens in AML can make a better discrimination of subpopulations of blasts, helping to perform studies of minimal residual disease [29]. It can also give prognostic information. The quantification of antigens in leukemia by mean fluorescence intensity (MFI), rather than percentage of positivity, provides an additional, distinct parameter by which to assess prognostic groups. Earlier studies have used the quantitative expression of CD34 and CD117 to detect MRD [33]. Authors in this study have correlated the intensity of CD117 with a decreased progression free survival and overall survival in patients with AML. They hypothesize that the intensity of CD117 may have increased significance for molecular targeted therapies for AML in the future. Defining and consistently using a particular monoclonal Ab, recognizing a specific antigenic epitope, prevents intra and inter-laboratory variation when studying the MFI values. Some studies have found that the three different epitopes of CD34 had variable quantitative expression in AML cases [34]. The binding of MoAbs was studied by flow cytometry, allowing evaluation of blast cell positivity as well as their mean fluorescence intensity. These quantitative data were made comparable between centers by means of calibration curve established with the same reagents in all laboratories. In cases of AML with t(9;22) the expression of class I CD34 was significantly higher than that of class II and III and the opposite was observed in AML with t(15;17). Moreover, as a whole, a high intensity of class III CD34 appeared to be a marker of good prognosis [34]. Since mAbs are commercially available from several sources with different clones that recognise different parts of the antigen with different avidities, a single source antibody is necessary for values consistency of MFI [5, 6].

The CD52 antigen is expressed on a majority of peripheral blood lymphocytes. Klabusay et al. [17] analyzed quantitatively the intensity of the CD52 antigen expression in patients with chronic lymphoproliferative diseases and compared it with B-lymphocytes values of a healthy population. In the group of patients with B-CLL, the CD52 level on tumor cells was significantly lower than on B-lymphocytes of the control group. By contrast, Rossmann et al. [35] have not found a statistically significant difference in the levels of CD52 between B-CLL patients and healthy donors. It seems possible that the small groups of patients and donors in this study could have led to inaccurate results. Furthemore, the cryopreservation used by this group have led to change in the antigen expression. Ginaldi et al. [36] had reported different results. In their study, the CD52 antigen levels in healthy donors as well as in patients with B-CLL vere lower than that in study of Klabusay et al. [17]. As with the study of Rossmann et al. [35], they did not note statistical differences between these two groups. In their study, however, all patients had been previously treated. The fact that those patients had been treated could account for the difference in comparison with the untreated patients in study of Klabusay. Large differences in results between authors can reflect a problem of sample preparation; FITC is very sensitive to pH change. The decline in pH leads to lower fluorescence of FITC, which can be seen mainly between pH 7 and 8. These data were confirmed in several assays, when fluorescence intensity of FITC-bound beads was measured in solutions of different pH value [17].

It was stated that leukemia cells could express after treatment some antigens of different density than normal cells, which might be used to detect antigen modulations after chemotherapy and to increase the accuracy of diagnosis by comparison with values in normal or by chemotherapy influenced counterparts [20]. Matutes and Polliack [37] described significantly higher antigen density of CD5 marker in majority of B-CLL cases comparing to that of control subjects. These quantitative studies could also help to monitor the numbers of residual malignant cells during and after therapy [38]. We suppose that quantitative immunofluorescence may be important for more precise and reliable leukemia diagnosis as well as for therapy strategy of this disease. The results described in the study Kusenda and Babusikova [21] showed the significance of the antigen density measurement in leukemic cells at diagnosis and after therapy too. They showed that chemotherapy treatment in B-CLL cells did not influence in significant level the antigen modulation of B-CLL cells in sense of their antigen expression. However, they observed that this treatment could modulate MESF values of some characteristic markers (CD5, CD19, CD20 and CD23) in B-CLL patients.

Sukova et al. [23] evaluated the CD20 density on tumor cell populations in patients and on the B-lymphocytes of the control group. In the patients with B-CLL, the CD20 density was low and it was significantly lower than in donors. The density of CD20 in mantle cell lymphoma (MCL) was high and may be helpful in differential diagnosis against B-CLL. The density of CD20 may play an important role in prognostic significance in patients with B-CLL [39]. However D'Arena et al. [40] had reported interesting results. The cells of some patients with B-CLL expressed the high density of CD20 antigen and these cells were found to be also morphological atypic. Huh et al. [41] and Babusikova et al. [42] described significantly higher density of CD20 antigen in patients with hairy cell leukemia (HCL) comparing to that of control subjects.

Matos et al. [43] evaluated the expression of some integrins on molecules on peripheral blood tumor cells of patients with B-CLL, MCL, and nodal or splenic marginal B-cell lymphoma, all in the leukemic phase and before the beginning of any therapy. The MFI of integrins was measured by flow cytometry of CD19-positive cells and differed amongst the types of lymphomas. Comparison of B-CLL and MCL showed that B-CLL presented a higher expression of CD11c and CD49c, and a lower expression of CD11b and CD49d. Comparison of B-CLL and marginal B-cell lymphoma showed that the former presented a higher expression of CD49c and a lower expression of CD11a, CD11b, CD18 and CD49d. Finally, comparison of MCL and marginal B-cell lymphoma showed that the latter had a higher expression of CD11a, CD11c and CD18. The finding of the lower expression of CD11a and CD49d in B-CLL suggests that these integrins molecules are probably responsible in the different compartment of the disease infiltrates compared to its related entity in the small lymphocytic lymphoma [44]. Zucchetto et al. [45] in their study demonstrated prognostic relevance for the combination of high CD49c and low CD49d expression in B-CLL. CD11b has a heterogeneous expression in B-CLL and may have other biological functions. It has been reported to be associated with a higher probability of disease progression and poor survival as well as to prevent the induction of apoptosis in this disease [46]. The role of CD11c in the disemination of B-NHL is still controversial [43]. Moreover, this antigen is steady over-expressed in hairy cell leukemia [42]. The results of study of Matos et al. [43] allow authors to conclude that B-CLL, MCL and marginal B-cell lymphoma have different adhesion molecule profiles and these differences may be responsible for the distinct capacities of these diseases to disseminate into the blood stream. These data reinforce the hypothesis that the peripheral blood invasion always present in B-CLL is the consequence of an imbalance of adhesion molecule expression in tumor cells. The authors demonstrated that the density of CD49c/CD49d pair consistently presented a distinct pattern of expression in B-CLL compared to MCL and marginal B-cell lymphoma, which could be helpful for the differential diagnosis.

The common leukocyte antigen, CD45, is a family of glykoproteins with phospho-tyrosine phosphatase activity, expressed on all cells of hematopoietic origin (except erythrocytes). CD45 seems to play an important role on the regulation of cell differentiation. CD 45 expresion varies during B-cell ontogeny [47]. B-cell precursors and hematogones show relatively low levels of CD45, while mature B-cells show higher surface CD45 density [15, 16].

CD45 is expressed by all non-Hodgkin´s lymphomas (NHL), but some differences in cell surface density of such a marker in the various subtypes have been reported [20]. In the study of Maljaei et al. [48] has been demonstrated that CD45 expression is lower in typical CLL when compared with atypical CLL and other NHL types. However very few cases have been included in the study. Carulli et al. [16] confirmed that lymphocytes from typical CLL showed very low levels of CD45 and that HCL lymphocytes were characterized by the highest CD45 density among the various subtypes of low-grade B-cell NHL. This study provides new information about CD45 expression in other types of NHL. Authors found that MCL lymphocytes display slightly higher levels of CD45 when compared with CLL lymphocytes and that intermediate values of CD45 expression are observed in the other NHL subtypes, such as lymphoplasmacytic lymphomas (LPL), marginal zone lymphomas (MZL) and follicular lymphomas (FL). Malignant cells may not always show the same CD45 and side scatter characteristics as their normal counterparts. These differences may by useful in identifying small populations of clonal cells. It appears that CD45 can by used to differentiate small numbers of leukemia hairy cells in peripheral blood and bone marrow, in absence of obvious morphological involvement. Hairy cells have a significantly higher mean cell fluorescence than normal lymphocytes [42], monocytes and CLL cells, but not all NHL cells. Since it is known that CD45 intensity increases in the course of B-cell ontogeny, data of these autors are consistent with different levels of maturation of the pathologic B-cells in such lymphomas. Measuring CD45 expression on lymphomatous lymphocytes could be useful to improve diagnosis in atypical cases.

Further studies are needed to evaluate the usefulness of these findings in the clinical situation. CD45 intensity measurement could be useful in cases of particular diagnostic difficulty, such as CD23-positive MCL, CD5-positive FL, leukemic phases of NHL mimicring CLL and CLL with atypical phenotype [16]. Significantly reduced expression of CD45 antigen is stable finding in plasmocytoma or myeloma cells [49].

The determination of antigen density (MESF values) is an important diagnostic marker of leukemic cells, suitable for study of antigen modulation analysis of some markers of leukemia cells in common and could be even a good model for the study of immunophenotype after treatment and for the study of antigen modulation caused by chemotherapeutic agents.

Quantitative fluorescence measurement has become increasingly important in the clinical laboratory as new immunotoxin therapeutic agents have been developed. Clinicians recently attempt to kill tumor cells by complexing toxins to an antibody that will recognize a specific tumor antigen and bind to it. Because response to therapy depends on levels of cell surface expression of the tumor antigen, quantitative fluorescence is necessary in evaluating patients for this treatment [2].

The measurement of antigen intensity expression is crucial to the interpretation of data and precise classification of some hematologic malignancies. Being able to consistently perform semiquantitative measurements has the high benefit in the most clinical situations. Such measurements will definitely be a standard part of the daily clinical laboratory setup for analysis of hematologic malignancies in the near future.

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