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Two methods for the quantitative analysis of surface antigen expression in acute myeloid leukemia (AML)

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Abstract: The expression of lineage molecules (CD13 and CD33), c-Kit receptor (CD117), CD34, HLA-DR and adhesion molecule CD49d was assessed in acute myeloid leukemia (AML) blast cells from 32 cases, using direct and indirect quantitative cytometric analysis. High correlation (r=0.8) was found between antigen expression intensity values calculated by direct analysis method (ABC) and by indirect analysis method (RFI). Moreover, the differences in expression intensity of CD13, CD117 and CD34 antigens were found between leukemic and normal myeloblasts. This may be helpful in identification of leukemic cells in the diagnostics of minimal residual disease after treatment in AML patients.

Key words: Antigen expression - Quantitative analysis - Myeloid markers - Acute myeloid leukaemia - Minimal residual disease

Introduction

In acute leukemias, abnormalities concern not only antigen expression but also its intensity, which may have diagnostic or prognostic significance [3, 5, 6, 8, 12, 14]. Hence, there is a necessity of accurate and reliable cytometric quantitative evaluation of surface antigen expression. Special indices were established to calculate relative "expression intensity" in relation to the type of cytometer and fluorescence intensity of isotypic control; an example of such index is so called relative fluorescence intensity (RFI) [5, 10]. Moreover, there are special kits for direct determination of the number of antibody molecules bound to a corresponding antigenic epitope which allow to assess capacity of antibody binding by means of an index called ABC - antibodies bound per cell [9].

Immunophenotypic characteristics of AML myeloblasts based on accurate qualitative and quantitative description of antigens are important for diagnosis. The literature data show that depending on the type of proliferation (acute or chronic) there are differences in the density of CD33 antigen on leukemic myeloblasts [7]. Our data show that the density of CD117 (c-Kit receptor) on bone marrow myeloblasts in AML patients depends on the number of white blood cells in peripheral blood [15].

The objective of our study was to perform a quantitative expression analysis of myeloid lineage antigens: CD13, CD33, CD117 and CD34 that occur on early blood progenitors and leukemic cells, by two quantitative cytometric methods: direct (ABC) and indirect (RFI).

Materials and methods

Patients. Bone marrow aspirates were collected from 32 patients with M1 or M2 adult acute myeloid leukemia. After informed consent and agreement of the Bioethical Board, 10 bone marrow aspirates (BM) were obtained from healthy adults serving as control subjects. AM diagnosis by FAB group criteria was established using morphological, cytochemical and immunophenotypical examination of bone marrow smears [1].

Cell preparation and immunofluorescence. Two-ml fresh heparinized bone marrow aspirates were diluted with an equal volume of PBS. Cells were incubated with the appropriate monoclonal mouse anti-human antibodies. Cell surface markers were assessed by direct immunofluorescence method, using phycoerythrine (PE)-conjugated monoclonal antibodies against antigens: CD13, CD33, CD34 and CD117PE (clone 95C3), HLA-DR and CD49d (Becton Dickinson, USA). 10 000 cells were counted and data were recorded in the list mode.

Flow cytometry studies. All samples were analysed using flow cytometers: CytoronAbsolute with ImmunoCount software (Ortho) and/or a FACS Calibur with CellQuest software (BD).

Electronic gating on the basis of FSC and SSC first eliminated cellular debris and nonviable cells. Distinct cell populations present

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Table 1. Comparison of surface molecule expression intensity on AML myeloblasts measured by two quantitative flow cytometry methods: antibodies bound per cell (ABC) and relative fluorescence intensity (RFI)

Antigens		CD13		CD33		CD34		CD117		CD49d	
Quantitative metods		ABC	RFI	ABC	RFI	ABC	RFI	ABC	RFI	ABC	RFI
AML M1+M2	Mean	7 232	14,8	4 549	12,4	14 607	17,7	10 615	16,5	4 677	13,7
	SD	5 684	3,8	3 542	3,3	11 941	3,5	6 163	2,1	2 209	2,1
	Min	614	7,1	1 266	6,1	2 059	11,1	3 209	12,5	1 536	9,4
	Max	21 117	21,4	12 273	20,0	62 234	24,0	25 349	20,4	11 865	17,3
Normal bone marrow	Mean	3 344	14,1	4 221	14,4	17 846	20,3	4 042	14,1	5 352	14,4
	SD	1 178	1,7	2 027	2,0	2 831	1,3	324	1,1	1 176	0,8
	Min	1 604	11,9	1 354	10,6	13 986	18,2	3 571	12,2	3 856	13,5
	Max	5 184	16,9	7 319	16,7	21 526	21,9	4 492	15,2	7 014	15,5

in the specimen were identified using CD45 and SSC. On the CD45/SSC dot plots, 4 gates were set up for lymphocytes, monocytes, myeloblasts, and granulocytes. To determine the immunophenotype of myeloblasts we used a triple labelling method with CD45-PE-Cy5 against each CD-FITC and CD-PE. FSC/SSC and CD45/SSC gating assessed antigen expression on myeloblasts and the threshold value for antigen positivity was more than 20% positive blasts.

The receptor expression was measured by an indirect quantification method previously described by Miwa *et al*. [10]. Antigen expression was quantified by measuring the mean value of PE fluorescence (in linear scale) of molecules and their isotype control on AML and normal bone marrow myeloblasts. The distribution of the fluorescence was always close to a normal Gaussian distribution; thus the mean value of the fluorescence histogram was a good representative parameter.

The intensity of antigen expression was found by relative fluorescence intensity (RFI) according to the following formula:

RFI = 10 × (3.5 × mean channel of antigen fluorescence ÷ number of channels) –10 × (3.5 × mean channel of isotype control fluorescence ÷ number of channels) [10]

Standardization was performed with fluorescent beads (Dako fluorospheres).

Direct quantitative analysis were completed by converting fluorescence intensity into the number of antigen molecules per cell, as measured by antibody binding per cell (ABC). The QuantiBRITE test (Becton Dickinson) was applied. The mean channels of PE fluorescence were found and ABC was then calculated, using QuantiCALC software [4].

Statistical analysis. Data are presented as mean ± SD and the interquartile range. Box-whisker plots illustrate the data distribution. Significance of differences was assessed by Student's t-test, using Statistica software. Differences were considered significant for $p<0.05$. Correlation coefficient was determined by linear regression.

Results

Comparison of results of quantitative expression analysis obtained by two methods: ABC and RFI

The first quantitative method for the analysis of antigen expression was the antibody bound per cell (ABC) method. In the majority of cases, the number of binding sites (ABC) for myeloid lineage markers CD13 and CD33 ranged from 1000 to 13000 and for CD117 and CD34 from 3000 to 20000. The most homogeneous density on the M1 and M2 leukemic myeloblasts was shown by adhesion molecule CD49d; in the majority of cases the number of antibody binding sites ranged from 4000 to 6000.

The second method of calculation was the relative fluorescence intensity (RFI) index according to Miwa's formula; an indirect method with regard to isotypic control fluorescence values. Data obtained using this method showed that the range of RFI values for CD13 and CD33 was wide, from approximately 8 AU (Arbitrary Units) to 20 AU while for CD117 and CD34 from 11AU to 22 AU. Detailed data are summarised in Table 1.

If antigen expression intensity was high, it was revealed by both methods (ABC and RFI). Likewise, low ABC expression intensity values corresponded to low RFI values. Correlation coefficient between ABC and RFI values was high: r = 0.7 - 0.8 (Fig. 1).

Comparison of the number of antibody binding sites (ABC) on leukemic and normal bone marrow myeloblasts

The ABC and RFI values for CD13 and CD117 on bone marrow myeloblasts in more than 60% of AML cases were higher than these found in healthy controls; only in small subset of AML patients (from 0 to 4%) the values were lower than those of the controls.

The pattern of expression intensity presented in ABC and RFI for CD33 and CD34 antigens was different: approximately 50% AML cases showed ABC and RFI values below the lower range for those antigens in normal myeloblasts. In 4 cases, CD34+ myeloblasts showed ABC and RFI values above the upper range value for normal bone marrow myeloblasts (Fig. 2).

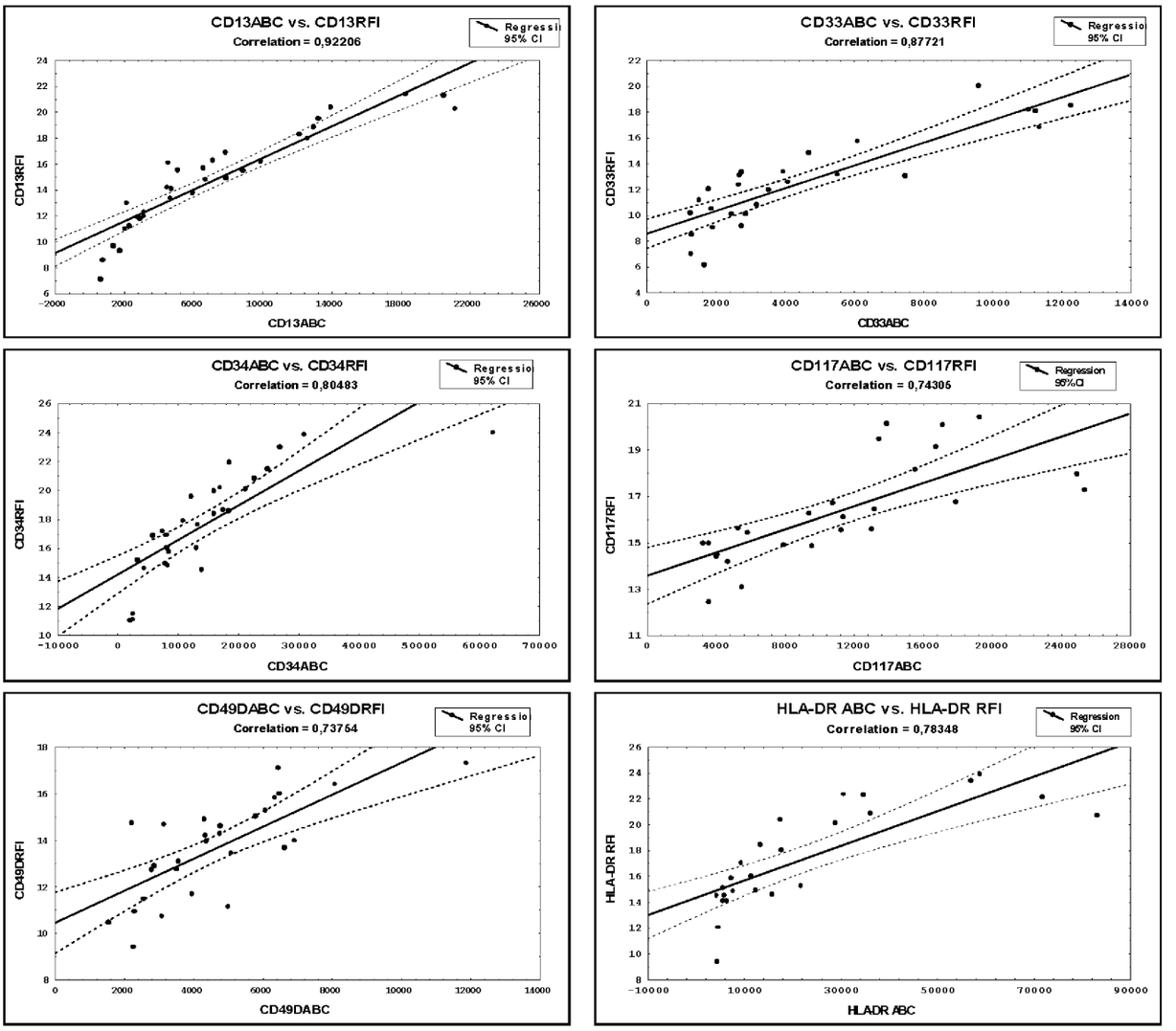

Fig. 1. Correlation between the values of expression intensity measured using two quantitative methods: ABC and RFI.

Discussion

Comparison of the values of CD13, CD33, CD34 and CD117 antigen expression intensity, represented as RFI, and these obtained by the ABC method shows high positive correlation. Such a correlation suggests that in the situation when object of interest shows differences in antigen expression intensity between leukemic blast cells and the normal bone marrow blast cells, a simple calculation of RFI index is satisfactory and there is no need to use reagent kits for the ABC determination.

In recent years, there is an increasing interest in applying new methods for the determination of minimal residual disease (MRD) in patients with AML. The cytogenetic examination is the most sensitive and reliable method, but it is difficult to perform and expensive. Another useful method is determination of detailed immunophenotype of leukemic myeloblasts with specific, atypical coexpressions. Unfortunately, in many cases the immunophenotype of leukemic cells is similar to that of normal myeloblasts [13]. A comparison of ABC results of myeloblast antigens, especially CD13, CD117 and CD34 shows differences in their density between leukemic and normal myeloblasts. Increase in CD13 density may be the result of regulatory role of this molecule in the angiogenesis; this process is unregulated in AML [2]. The increase in c-Kit receptor (CD117) density may be related to the changes in adhesive properties of leukemic myeloblasts leading to leukopenia in these patients. [11].

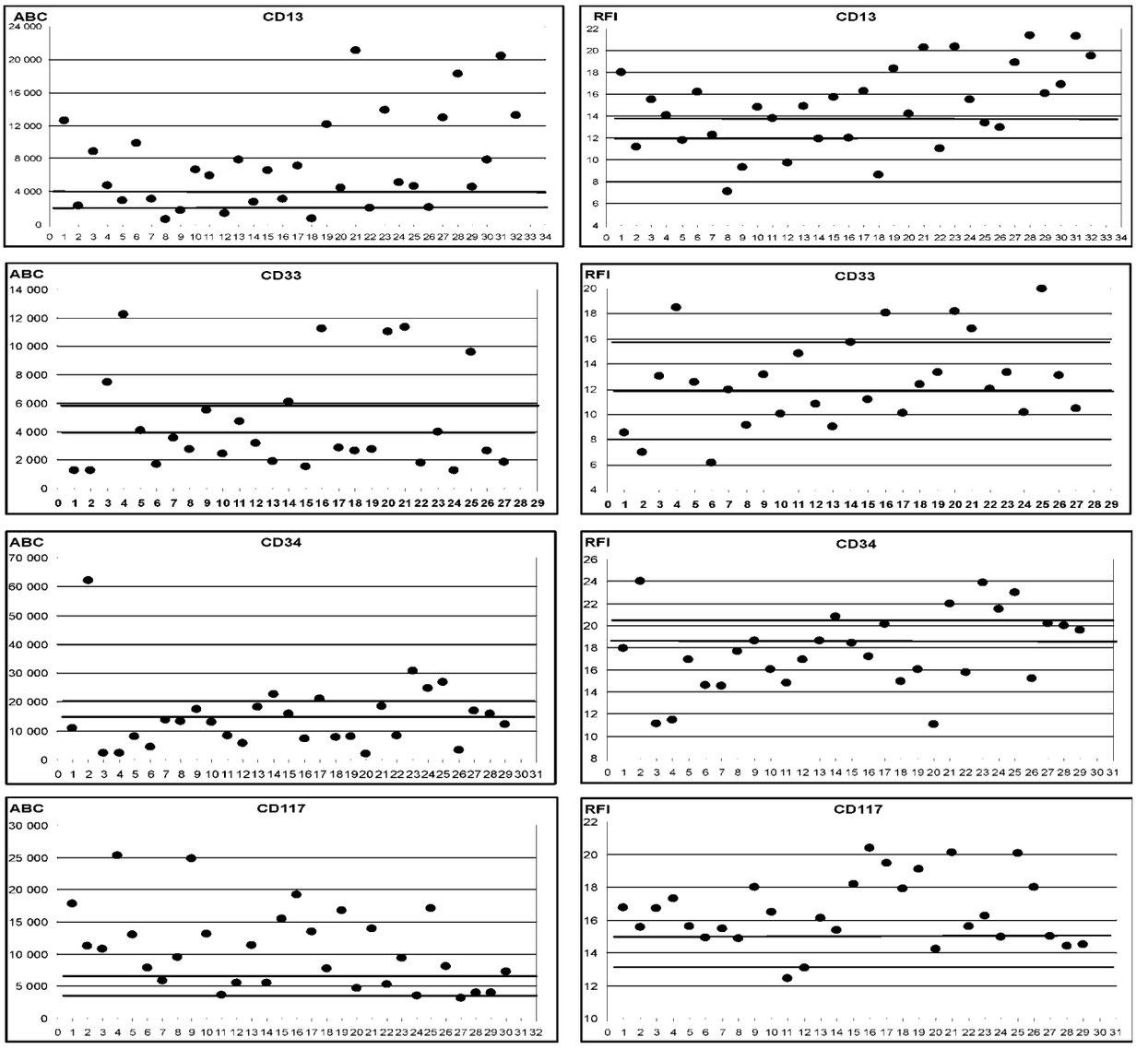

Fig. 2. ABC and RFI values of surface molecules on AML and normal bone marrow myeloblasts. Each point indicates an individual patient. Thick parallel lines indicate range of values for normal bone marrow myeloblasts.

In summary, it has been demonstrated in our study that determination of expression intensity may be a useful tool for diagnosis and a specific, individual panel of surface antigens with special regard to quantitative characteristics of antigen expression can be evaluated for each patient. This panel should be further applied to the identification of leukemic cells in investigation of minimal residual disease.

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