Changes in antigen expression on B lymphoblasts of acute leukemia may facilitate recognition of minimal residual disease

WIOLETA ZALEWSKA[1], ELŻBIETA GÓRSKA[1], AGNIESZKA OBITKO-PŁUDOWSKA[2], MARIA WĄSIK[1]

[1]Department of Laboratory Diagnostics and Clinical Immunology of Developmental Age, Medical University of Warsaw, Poland
[2]Department of Pediatrics, Hematology and Oncology, Medical University of Warsaw, Poland

Abstract

In the last decade the introduction of new drugs and protocols of treatment, together with very precise diagnoses based on the flow cytometric assessment of immunophenotype leukemic blasts, have significantly improved the survival of patients . The leukemia treatment is considered successful when bone marrow is free from leukemic cells. Even if only one neoplastic cell survives in the patient's bone marrow, the disease can relapse. For this reason it is necessary to be sure that counted blasts in bone marrow are neoplastic cells. The aim of the study is to assess the advantages of the measurement of surface antigens expression that allows drawing the distinction between leukemia blasts and normal cells. We concluded that expression of CD45, CD10 and CD184 on leukemic blasts is different than on normal bone marrow cells and that the measurement of relative fluorescence intensity (RFI) for 2 or more antigens would simplify the search for leukemia blasts in bone marrow side population.

Key words: *cALL, flow cytometry, relative fluorescence intensity.*

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Introduction

Acute B-lymphoblastic leukemia is one of the most common childhood malignancies. In the last decade introduction of new drugs and protocols of treatment, together with very precise diagnoses, based on the flow cytometric assessment of the immmunophenotype of leukemic blasts, have significantly improved survival rates of patients [1-3]. Flow cytometry and a widely available set of monoclonal antibodies allow the recognition of the origin of leukemic cells and the stage of their differentiation [4, 5]. The most common acute childhood leukemia concerns B-lymphocytes in an early stage of differentiation with blasts expressing the antigens CD19 and CD10 [6]. If the blasts have any atypical surface antigens, it is very difficult to assess the effectiveness of anti-leukemia treatment because normal B-lymphocytes with an expression of CD10 are also present in the bone marrow of healthy subjects. For this reason, studies should be undertaken to look for characteristic features that will allow to distinguish, with high probability, normal cells from malignant cells.

The probability of correct recognition of malignant cells has increased with rising knowledge of these differences owed to flow cytometric analysis. It is possible to prepare appropriate protocols and separate the group of cells in which the presence of malignant cells is suspected. Leukemia treatment will be successful when the bone marrow is free from tumor cells. Even if only one neoplastic cell survives in the patient's bone marrow, the disease can relapse. For this reason, it is very important to look for new features that increase the prognostic value of results suggesting complete remission [6]. It is widely known that stem cells, leukocytes and platelets possess surface receptors (CXCR4 – CD184) for chemokine (CXCL12 – SDF-1), constitutively synthesized by bone marrow stromal cells and other cells of various tissues. Lack of or low expression of CXCR4 or a defect of stromal cells in the

Correspondence: Wioleta Zalewska, Department of Laboratory Diagnostics and Clinical Immunology of Developmental Age, Medical University of Warsaw, Marszałkowska 24, 01-452 Warsaw, phone number: 508 653 456 or phone number/fax: +48 22 629 65 17, e-mail: wiola.zalewska@wp.pl or wasik@litewska.ed

production of its ligands can be responsible for the migration of leukemic cells outside of bone marrow and infiltration into other organs [7].

The aim of the research is to assess the advantages of measuring the expression of the surface antigens that allows drawing the clear-cut distinction leukemia blasts and normal cells. For this purpose, when conducting the cytometric assessment of immunphenotype malignant blasts, we have decided to take into consideration not only the percentage of cells with the expression of characteristic surface antigens, but also their intensity of fluorescence as the indicator of the density of these antigens.

Materials and methods

Patients

The study was performed on a group of 10 children (6 boys and 4 girls, mean age 3,7 ±0,31 years) with recognized acute leukemia, on the basis of morphological and cytochemical examination, as well as flow cytometric analysis. For assessment of blasts phenotype, bone marrow aspirates (0,5 ml) were taken into tubes containing EDTA as an anticoagulant. Common B-lymphoblastic leukemia (CD19+D10+) was diagnosed in all children participating in this study. From the day of diagnosis (day 0), children were treated according to program ALL IC-BMF 2002, protocol I, phase I (induction therapy), which included Predrisone, Vincristine, Daunorubicine and L-asparaginase. After 15 days, the bone marrow was again taken to be examined for lymphoblasts and the percentage of bone marrow lymphocytes and their antigens expression.

Staining procedure

The number of nuclear cells in bone marrow tissues was assessed using haematological analyser and adjusted to the concentration of 5×10^6/ml in PBS. For recognition of the type of leukemia, 100 µl of bone marrow cells were transferred to tubes and 10 µl of following monoclonal antibodies (mAbs) against CD45, CD2, CD3, CD5, CD7, CD4,CD8 CD10, CD19, CD20, CD13, CD14, CD15, CD33, CD34, CD79a, CD184, TdT, HLA-DR, κ, λ, IgG and IgM directly labeled with fluorochrome (FITC, PE, PC5) were added. 3-4 antibodies conjugated with different flourochromes were added to one tube. All staining procedures were performed according to the instruction attached to every monoclonal antibody. In all experiments, cells were stained with the appropriated isotopic control.

Flow cytometric analysis

The 10 000 cells in every tube were analysed in a FC500-5C flow cytometer (Beckman Coulter, USA). Electronic gating on the basis of FS/SS allowed the elimination of cellular debri and non-viable cells and SS/CD45 allowed the elimination of high granular cells CD45+. The results were obtained as a percentage of positive cells in analysed gates. Relative fluorescence intensities (RFI), were calculated using

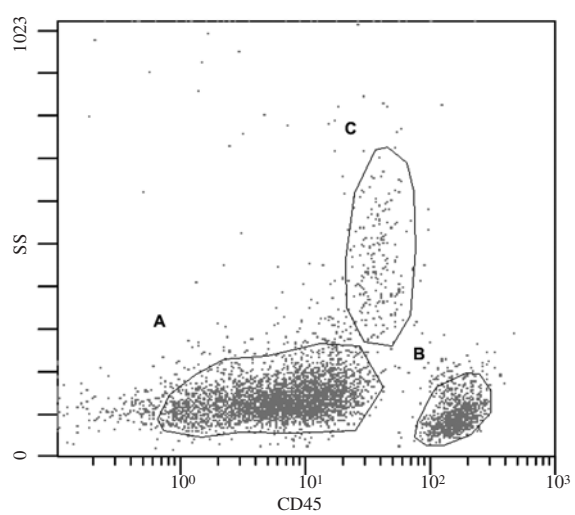

Fig. 1. Typical cytogram of bone marrow cells obtained from the patients diagnosed with acute lymphoblastic leukemia. Dot plots represent the standard method of gating according to SS (side scatter) axis and CD45FITC fluorescence. Gate A: blasts cells CD45 dim, gate B, lymphocytes CD45 bright, and gate C; neutrophils CD45 intermediate

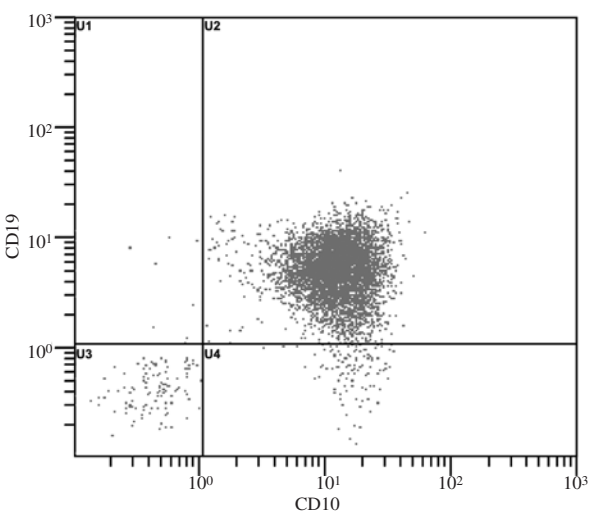

Fig. 2. Examination of lymphoblasts gated in Fig 1. as A. according to the expression of CD19FL2 and CD10FL1 molecules. Dot plots U2 show positive staining for CD19 and CD10 of the examined blasts. This analysis next became a basis for cell recognition. Simultaneously, the line to axes X and Y was created according to the bone marrow cells staining with isotypic control. The U2 region contains CD19 and CD10 positive blasts, whereas U3 area contains double negative cells. U1 region contain B-lymphocytes CD19+ CD10– while U4 contain CD10+ CD19– cells

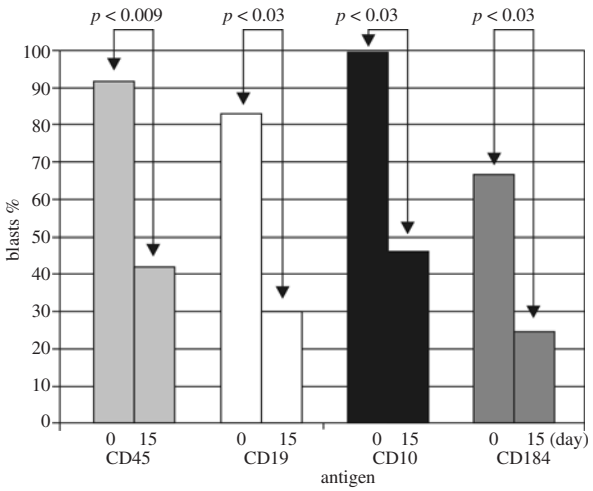

Fig. 3. The influence of anti-leukemic treatment on the percentage of bone marrow lymphoblasts (cALL-B) expressing the characteristic surface antigens. The number of lymphoblasts was measured at the day 0 (day of leukemia diagnosis) and compared to the number of blasts observed after 15 days of treatment. Statistical significance values are given at the top of figure

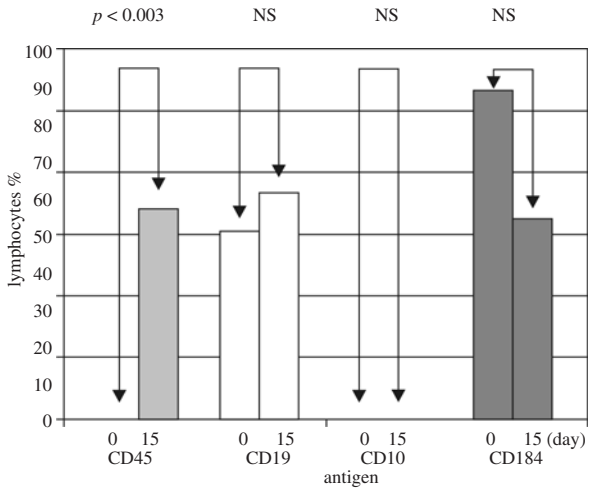

Fig. 4. The influence of anti-leukemia treatment on the percentage of bone marrow B-lymphocytes expressing the characteristic surface antigens. The numbers of lymphocytes was measured at the day 0 (day of leukemia recognition) and compared to the number of cells observed after 15 days of treatment. Statistical significance values are given at the top of figure

the formula: experimental mean fluorescence intensity (MFI)/MFI for with isotope control antibody as was described by Dechant *et. al.* [7].

Statistical analysis

The results were computed using the Statistica 7.1 programme (Statsoft, Poland). Mean results and standard deviation (SD) was calculated. Groups of results were compared by nonparametric methods, using the Mann-Whithney U-test. The significance level was determined as $p < 0.05$.

The study was approved by the Ethical Review Board of the Medical University of Warsaw.

Results

As shown in Fig. 1 of the flow cytometric analysis, children's bone marrow suspected of acute leukemia shows a mean presence of 89,5 ±34,21% of cells with a low surface number of CD45 molecules (CD45 dim), typical for leukemic blast cells (Fig. 1. gate A). The number of blasts were confirmed by microscope examinations. Only 6,7 ±1,32% cells possessed a high expression of the CD45 antigen (lymphocytes with CD45 bright, Fig. 1; gate B). In gate C only a few neutrophils were present (0,95 ±0,76%). In gate A (CD45 dim) 84,5 ±7,02% blasts show a typical expression for cALL of CD19+CD10+ (Fig. 2). Under the influence of anti-leukemia treatment the number of CD45dim blasts significantly sloped down, and a lower number of CD45dim cells were positive for CD19, CD10 and CD184 antigens (Fig. 3). At the same time, a significantly increased number of CD45 bright was observed, but the percentage of CD19, CD10, CD184 positive cells remained on the same level (Fig. 4). Changes of relative fluorescence intensity (RFI) examined after staining with monoclonal antibodies on day 0 and after 15 days of antileukemic treatment are presented in Table 1.

The RFI for CD45 measured on the blasts and lymphocytes on day 0 was highly different statistically (Table 1). The RFI of lymphocytes was about fifteen times higher than that of blasts. These differences were seen after 15 days of treatment as well, but the RFI of CD45 increased twice on the15th day. In the population of lymphocytes, any difference in the RFI of CD45 was observed, similarly to the RFI of CD19. The high RFI of CD10 observed on blasts on the day 0 was ten times lower on the day 15. On the day 0 the RFI of CD184 positive blasts was significantly lower in comparison to RFI B-lymphocytes. The number of CD45bright + CD184+ lymphocytes decreased after 15 days of antileukemic treatment (Table 1).

Discussion

The gradual intensification of chemotherapy with additional drugs improved the cure rates of leukemia patients. In the last decade, ALL is curable in more than 70% of cases. However, complete remission, defined in the examination of bone marrow morphology as presence of less than 5% of blasts, is not very precise, and in 30% of children, the disease relapse even after a few years. On the

Table 1. The comparison of changes of relative fluorescence intensity (RFI) of antigens examined on the surface of B leukemia blasts (CD45 dim) and B-lymphocytes (CD45 bright) in patients' bone marrow at the day of cALL diagnosis (day 0) and 15 days after beginning of the treatment

Antigen	Expression	Mean RFI ± SD		P-value
		Day 0	Day 15	
CD45	CD45 dim (blasts)	20.9 ±18.12	42.1 ±28.31	NS
	CD45 bright (lymphocytes)	327.1 ±111.41	344.3 ±142.24	NS
	P-value	0.0001	0.0008	
CD19	CD45 dim (blasts)	20.0 ±17.08	31.3 ±30.01	NS
	CD45 bright (lymphocytes)	25.5 ±14.26	18.3 ±13.34	NS
	P-value	NS	NS	
CD10	CD45 dim (blasts)	249.1 ±141.03	25.01 ±12.13	0.02
	lymphocytes	23.3 ±12.31	9.7 ±3.11	NS
	P-value	0.0003	0.01	
CD184	CD45 dim (blasts)	11.7 ±5.21	13.1 ±6.21	NS
	CD45 bright (lymphocytes)	23.2 ±5.03	11.3 ±4.21	0.03
	P-value	0.001	NS	

NS – non-significant

other hand, there are suggestions that the same children are overtreated. For both reasons, monitoring the response to treatment by periodic bone marrow examination comprise an integral part of the clinical monitoring the response of patients with recognized ALL [3, 8]. It is general understanding that one residual leukemia cell per 1000 cells in the bone marrow at the end of induction indicates a poor prognosis for the patient [5]. Flow cytometry analysis of a large number of cells, in a short time, allows the finding of even fewer cells whose phenotype differs from that of normal cells. In this quicker and cheaper method than molecular methods, results are obtained within an hour of sampling [3, 9]. In cases of cALL, a significantly different expression of CD45, CD10 and CD184 on B-malignant blasts in comparison to normal lymphocytes was found when the results were expressed as relative fluorescence intensities (RFI). It is our preliminary communication concerning the use of flow cytometry for the detection of the sensitivity of bone marrow cells to anti-leukemia treatment. Anti-leukemia treatment decreased the number of bone marrow cells with a low number of CD45 molecules and decreased the synthesis of CD10 and CD184 molecules. An assay performed on the 15th day shows less cells with RFI characteristic for leukemia blasts and increased cell number with RFI of normal B-lymphocytes. Our study group was too small for individual correlation of the results obtained with a clinical outcome, but we are planning to conduct a similar analysis for a higher number of patients monitored on days 15, 33 and 78 after leukemia recognition, when RFI changes will be compared not only for surface, but also for nuclear antigens.

At present, we can conclude that the results presented using a 5-colour flow cytometer suggest that it is better to consider not only changes of cell percentages with a leukemia phenotype, but performed analysis of RFI for typical antigens that were the basis of leukemia type recognition. If RFI will be analysed for two or more antigens, it would be easier to find leukemia blasts in the bone marrow repopulated by cells belonging to different lines, in different stages of development. For example, in the bone marrow of healthy subjects there could be up to 25% of B lymphocytes with expression of CD10. Examination of RFI for CD10 molecules may facilitate recognition of normal B-lymphocytes with low RFI of CD10 from leukemic B-lymphoblasts with high RFI of CD10 (see Table 1).

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