Multiparametric flow cytometry in detection of minimal residual disease in acute lymphoblastic leukemia of early B-cell phenotype*

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Received July 7, 2003

Leukemic cells can be distinguished from normal hematopoietic cells on the basis of chromosomal or molecular abnormalities, antigen receptor gene rearrangements and immunophenotype. Set of 3-, 4-combination of monoclonal antibodies was used for exact definition of immunophenotypic characteristics of B-cells populations from healthy donors and aberrant, asynchronous, over/under-expressed phenotypes and detection changes in intensity expression of markers that characterized pathological leukemic B-cells at diagnosis. These differences in normal and abnormal cell patterns were very important and could be utilized for analysis of minimal residual disease. On the basis of these findings we were able to clearly distinguish residual leukemic cells from hematogones (healthy B-lymphocyte precursors) too. We also verified that in some cases the CD58 marker is overexpressed on $CD10^+$, $CD34^+$ blast cells at diagnosis and can be feasible used for detection of minimal residual disease (MRD).

Key words: Multiparametric flow cytometry, acute leukemia, minimal residual disease.

The phenotypic similarities between the neoplastic cells and their normal counterparts were the basis for the classification of B-ALL. Nevertheless, a precise definition of the pathological hematopoietic cells is essentially based on the ability to clearly distinguish them from the healthy cells present in the specimen [13].

The knowledge of immunologic profiles of lymphoid cell subsets in normal bone marrow makes it possible to determine the aberrant phenotypes at diagnosis and to choose the marker combinations to be applied in the follow-up samples [1, 6]. Monitoring minimal residual disease (MRD) means identification of residual leukemic blasts resistent to chemotherapy and persisting in bone marrow or peripheral blood of patients after therapy. Detection of low levels pathological blasts by flow cytometry appears one of the most useful approaches to predict relapse and to discriminate patients with different relapse-risk [1, 6, 18].

For productive detection of MRD in B-ALL, it is necessary to clearly distinguish leukemic lymphoblasts from their normal counterparts, the B-lymphoid progenitors, so-called 'hematogones', that normaly reside in the bone marrow [5]. Hematogones are reported to occur in small numbers in some healthy children, may be prominent in the regeneration phase following chemotherapy or bone marrow transplantation and in patients with autoimmune or congenital cytopenias, neoplasms and AIDS [10]. Single- and two-color flow cytometry does not reliably differentiate hematogones from leukemic lymphoblasts. However, appropriately applied 3- and 4-color multiparameter flow cytometry is reported to distinguish between these cell populations in nearly all cases [3, 8, 9]. The study of the CD58 marker expression in ALL patients in clinical remission by Chen at al [5] indicates that large-scale gene profiling can be applied to identify new markers for monitoring residual cancer cells.

In the present study, 3- and 4-color flow cytometry was first used for precise characterization of the phenotypic profiles exhibited by B-cell population from healthy donors. In the next step, these profiles were used in the diagnosis of B-ALL as a template for identification of aberrant phenotypes of the blast cells and exact differentiation of hematogones

*This work was partly supported by the Grant No 2/5120/23 from the Slovak Grant Agency.

from persisting pathological B-cells after chemotherapy in the bone marrow or peripheral blood of the patients.

Material and methods

Patients and samples. Twenty-one patients with B-ALL were included in this study, 18 children and 3 adults. The median age of the patients at diagnosis was 5 years (range 1–59 years), and there were 9 females and 12 males. Eight patients (38%) had a leukocyte count less than $20x10^9$/l at presentation. Patients were treated according to ALL IC-BFM protocol.

Heparinized peripheral blood and bone marrow samples of these patients at initial diagnosis and during disease duration were used and the results have been stored at electronic form to compare the phenotypic patterns of leukemic cells over time, or to assess the individual leukemia-associated phenotypic aberrations. The control samples of PB or BM were obtained from healthy donors. Multiparameter analysis of lymphocytes was performed without isolation of cells on density gradient, using a commercially available red cell lysing solution (Optilyse B, Immunotech, France). The labeling cascade, cellular fixation/permeabilization procedures and dilution experiments have been described in detail [1, 7, 15].

Flow cytometric analysis. All antibodies directly conjugated by fluorochromes were used to specifically profile B-cell precursors and further cells of BM compartment: CD10 (5-1B4-R-PE) from Caltag, CA, USA, CD19 (J4.119-PE, -PC5), CD20 (B9E9-FITC), CD22 (SJ10.1H11-FITC), CD34 (581-PE), CD38 (T16-FITC), TdT (pool-FITC), CD45 (J33-ECD, -PC5), CD58 (AICD58-FITC), CD7 (8H8.1-FITC, -PE), CD13 (SJ1D1-FITC), CD33 (D3HL60.251-PE), CD11b (Bear1-FITC) from Immunotech, Marseille, France. Three-, four-combinations of monoclonal antibodies were used in standardized protocols at diagnosis: CD20-FITC/CD10-PE/CD45-ECD/CD19-PC5, CD22-F/CD34-PE/CD45-ECD/CD19-PC5, CD38-F/CD34-PE/CD45-ECD/CD19-PC5, CD58-F/CD10-PE/CD45-PC5, nTdT-FITC/CD10-PE/CD45-PC5. Standardized combinations of monoclonal antibodies which detect aberrant phenotypic features were used to detect MRD.

Measurements of antibody labeling were performed by multiparameter flow cytometry using an EPICS ALTRA flow cytometer equipped by Expo 32 program for analysis. At diagnosis, 30 000 events were evaluated. For MRD measurement, 30 000 events from total PB or BM were acquired first; in the second step a live gate was established on the basis of markers expression (CD19 or CD10 and side scatter characteristic, SSC). A minimum of 100000 cells were analyzed for each monoclonal antibody combination.

Results

Characterization of the phenotypic profiles exhibited by B-cell population from healthy donors. Multiparametric flow cytometry for precise characterization of the phenotypic profiles that expressed B-cell population of healthy donors was applied. In samples from healthy donors predominantly mature B-cell population with markers $CD45^{bright}$, $CD19^{+}$, $CD20^{bright}$, $CD22^{+}$, $CD38^{-/dim+}$, $CD34^{-}$, $CD10^{-}$, TdT^{-} markers was detected (Fig. 1). Other, less mature population of B-cells characterized by markers expression $CD45^{+}$, $CD19^{dim}$, $CD10^{+}$, $CD38^{+}$, $CD34^{+}$ was presented in very low proportion ($0.08 \pm 0.02\%$ of total bone marrow).

Diagnosis of early B-cell ALL and identification of aberrant phenotypes. A total of 58 patients were analyzed by standardized 3- or 4-color combinations of MoAb for suspect acute leukemia. On the basis of immunophenotypic profiles and comparisons of the healthy B-cell and blast populations from patients, 21 of them (36%) were defined as B-cell ALL.

Pathological leukemic cells (visualized on the dot plots in Fig. 2) exhibited phenotypes which reflect incomplete maturation and immunophenotypic aberrancy. These changes were different from that of normal B-cells with complete B-lineage maturation. We were able to detect the presence of at least two distinct pathological phenotypic patterns in each patient. Summary of the aberrant antigen expressions is detailed in Table 1.

Detection of MRD and differentiation of hematogones from pathological B-cells. After diagnosis, bone marrow samples were evaluated for MRD during and after treatment. The multiparametric analysis showed, that samples from the end of induction phase of treatment contained almost exclusively mature B-cells and T-lymphocytes. Their pattern was quite similar to the picture of healthy control donors. In contrast, in later phases (3–5 months after diagnosis) in some BM samples immature B-cells, so-called hematogones predominated (Fig. 3). Hematogones expressed phenotypic profiles that characterized normal B-lineage precursors. Fourteen of the 38 BM specimens contained at least 5% (from 5 to 27) of hematogones. These observations are similar to those presented in the BM samples during treatment in two AML patients, and did not represent either pathological clone or secondary malignancy (data not shown). All our patients in which hematogones were detected are still in complete remission (median duration 16 months, range 2–23).

In one patient with early B-cell ALL during relapse the pathological cells with phenotype profile: $CD10^{+}$, $CD19^{+}$, $CD34^{+}$, $CD38^{+}$ were found and a small part of the pathological clone expressed CD20 at very low intensity. After one

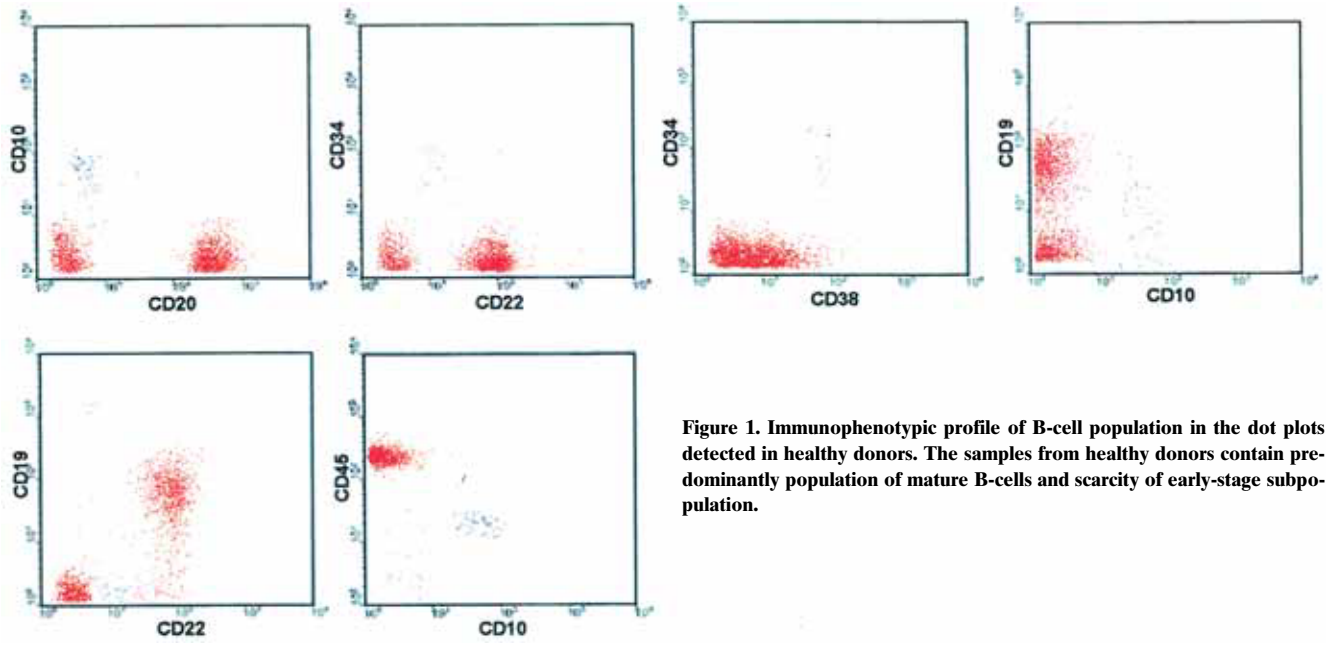

Figure 1. Immunophenotypic profile of B-cell population in the dot plots detected in healthy donors. The samples from healthy donors contain predominantly population of mature B-cells and scarcity of early-stage subpopulation.

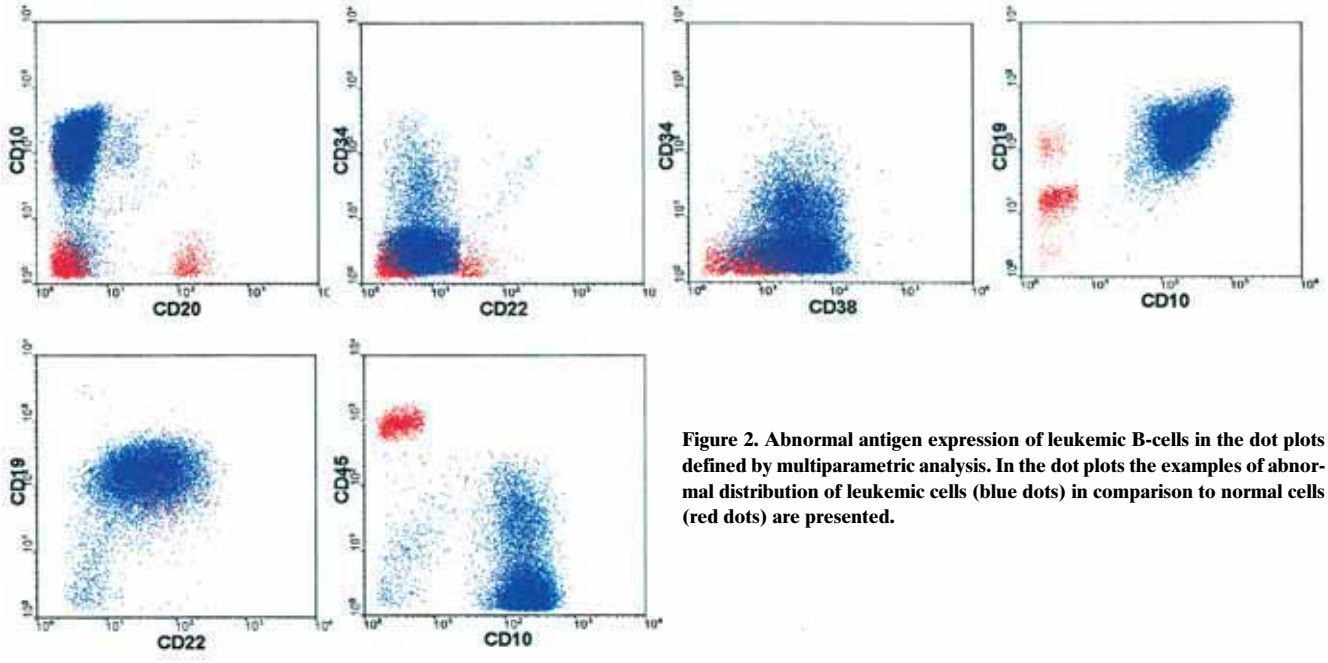

Figure 2. Abnormal antigen expression of leukemic B-cells in the dot plots defined by multiparametric analysis. In the dot plots the examples of abnormal distribution of leukemic cells (blue dots) in comparison to normal cells (red dots) are presented.

month MRD of a little different phenotype (CD20-positivity and CD34-negativity) was observed (Fig. 4).

CD58 as a marker for MRD detection. In some patients the expression of CD58 as a new possible marker of MRD detection in triple-marker analysis was followed and in some of them the coexpression of CD58 with CD10 and/or CD34 was found. The patterns found at diagnosis, MRD detection and relapse are shown (Fig. 5a–c). In majority of treated patients of early B-cell phenotype, the CD58 marker expression in MRD and hematogone detection was missing (Fig. 5d, 5e).

Discussion

Differentiation of hematopoietic cells from early commited progenitors to mature stages is a multistep maturation process that can be monitored by the coordinated acquisition and loss of leukocyte differentiation antigens [8]. More re-

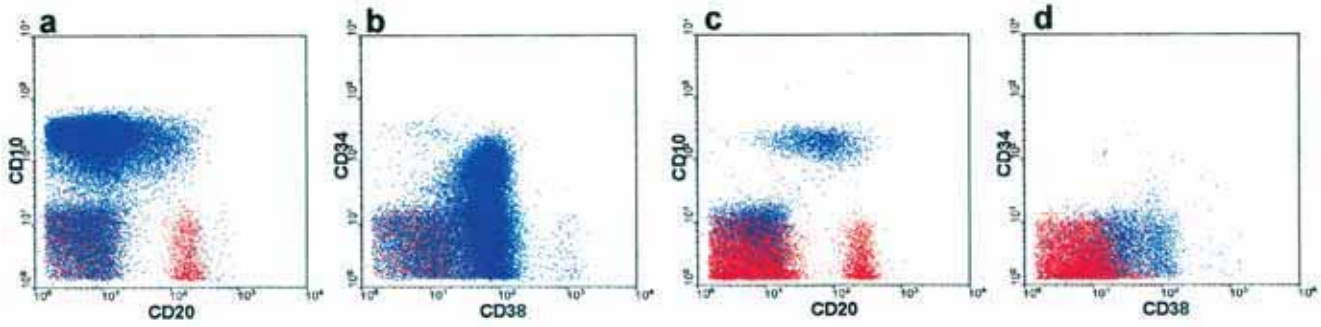

Figure 3. Flow cytometric dot plots showing examples of MRD detection. Plots a and b show antigen expression of leukemic cells represented as blue dots and residual mature B-cells (depicted in red). Plots c and d displayed detected MRD in the same patient.

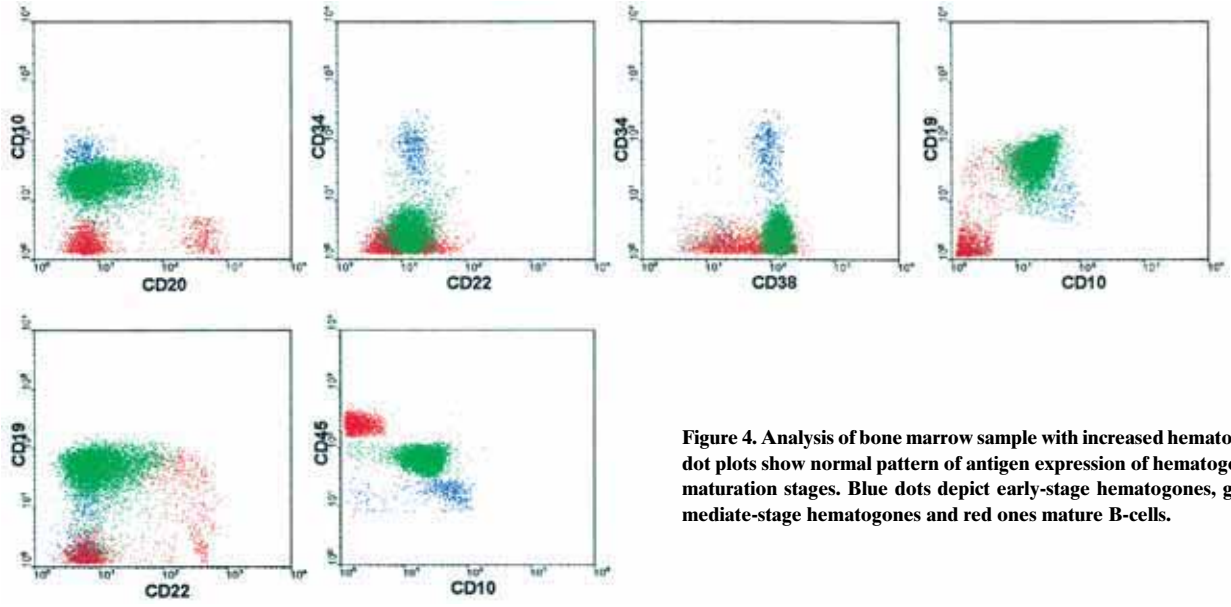

Figure 4. Analysis of bone marrow sample with increased hematogones. The dot plots show normal pattern of antigen expression of hematogones in two maturation stages. Blue dots depict early-stage hematogones, green intermediate-stage hematogones and red ones mature B-cells.

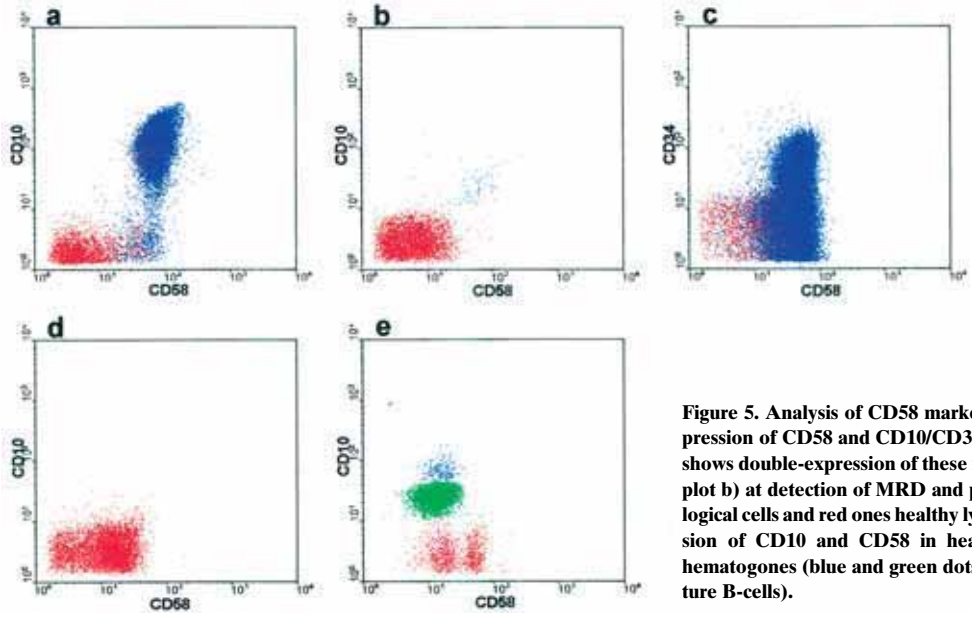

Figure 5. Analysis of CD58 marker expression. The dot plots illustrate expression of CD58 and CD10/CD34 in normal and leukemic B-cells. Plot a) shows double-expression of these markers in cells from patient at diagnosis, plot b) at detection of MRD and plot c) at relapse (blue dots depict pathological cells and red ones healthy lymphocytes). Plots d) and e) show expression of CD10 and CD58 in healthy donor and patient with increased hematogones (blue and green dots indicate hematogones and red ones mature B-cells).

Table 1. Aberrant antigen characteristics detected in BM from 21 patients with B-ALL

		No. of patients (%)
Aberrant antigen expression		
CD45	negative or underexpressed	6 (29%)
CD10	negative	2 (10%)
	overexpressed	8 (38%)
CD19	underexpressed	4 (19%)
	overexpressed	5 (24%)
CD34	overexpressed	3 (14%)
CD20	overexpressed	2 (10%)
CD22	negative	1 (5%)
	overexpressed	3 (14%)
CD38	underexpressed	4 (19%)
TdT	uniform expression	2 (10%)
Cross-lineage antigen expression		
CD33		2 (10%)
CD11b		1 (5%)
CD7		1 (5%)
Aberrant antigen coexpression		
$CD45^{dim/-}/CD34^{dim/-}$		4 (19%)
$CD10^{bright}/TdT^{dim+}$		2 (10%)
$CD10^{dim/-}/CD34^{+}$		3 (14%)
$CD10^{bright}/CD20^{bright}$		1 (5%)
$CD10^{dim/-}/TdT^{dim/-}$		1 (5%)
$CD34^{+}/CD22^{bright}$		2 (10%)

cently, several studies have been directed to the investigation of cell populations present in bone marrow samples from healthy donors [7, 14]. These studies, using multiparameter flow cytometric analysis have focused on the identification of B- and T-cell subpopulations present at low frequency in normal bone marrow. A part of our study reports the findings of multiparametric analysis of bone marrov samples in healthy individuals. We characterized here the immunophenotypic profile of predominantly more mature B-cell population with immunophenotype $CD45^{bright}$, $CD19^{+}$, $CD20^{bright}$, $CD22^{+}$, $CD38^{-/dim+}$, $CD34^{-}$, $CD10^{-}$, TdT^{-}. However, Lucio et al [7] identificated 3 distinct subpopulations of $CD19^{+}$ precursor B-cells. This difference might be caused by bone marrow samples obtained from BM donors for transplantation, where early hematopoietic progenitors could be present expressing B cell-associated antigens.

By the method of single- or double-staining it was possible to characterize leukemia clone and some of its aberrant characteristics. It was possible to define the cross-lineage antigen expression (expression of B- or T-cell marker in myeloid leukemia cells and *vice versa*), asynchronous antigen expression (the coexistence of two antigens in the same cell that are not simultaneously expressed in the normal differentiation) and abnormal light-scatter patterns. Multiparametric flow cytometry by specific marker combinations makes it possible to distinguish very exactly the pathological clone from normal cells (by their complex definition in the so-called 'empty spaces', i. e. in places in which normal cells are absent). The specific aberrant patterns of antigen expression in 21 patients with B-ALL have been characterized. We found that in each patient at least two distinct pathological phenotypic characteristics of leukemic cells were present and the patterns found were suitable for MRD detection.

In addition to detection of MRD, in part of our treated patiens a relatively large number of hematogones (immature progenitor B-cells) in bone marrow samples repeatedly were detected. It is known that in some clinical settings (e.g. following treatment) hematogones often expand in regenerating bone marrow and can pottentialy be mistaken for residual disease [10]. However, hematogones can be reliably distinguished from pathological cells by multiparametric flow cytometry, as the pattern of antigen expression in normals is very reproducible and allows to create a fixed set of regions to define the normal progenitors [4, 19].

Flow cytometric immunophenotyping for MRD detection has generally been accepted as one of the most useful approaches to predict relaps and to discriminate high-risk patients. Studies based on semiquantitative polymerase chain reaction (PCR) or immunophenotyping demonstrated clinical significance of MRD detection. These results show that measurements of MRD by flow-cytometric method and by PCR assay are comparable and that levels of MRD associated with a higher risk of relapse (i. e., $>10^{-4}$) can be detected by either technique [12, 18]. Early response to therapy is one of the most, if not the most, important independent prognostic factor in acute leukemia.

One of the difficulties in flow cytometric application for MRD studies is the lack of leukemia-specific markers. In BM sample from one our B-lineage ALL patient after relapse the pathological cells of a little different immunophenotypic profile characterized by a loss of CD34 and expression of CD20 by all pathological cells were detected; other phenotypic characteristics remained stable. As we presented in our previous study, minor immunophenotypic shifts represented by loss or acquisition of some markers are frequently observed in B-ALL patients [17]. Clonal evolution or clonal selection is a relative disadvantage not only for MRD detection by flow cytometry, but also PCR amplification of Ig and TCR genes [3, 16].

Investigation of adhesion molecules on blast cells of B-ALL cases revealed that they expressed adhesion molecules at high rates [11]. Chen et al [5] identified 7 proteins, including CD58, expressed in B-lineage ALL cells at higher levels than in their normal counterparts. The CD58 antigen was chosen for further analysis. It was also tested in our study, whether this marker was overexpressed in all newly diagnosed cases of B-ALL and might have become independent marker for reliable detection of MRD. In some cases CD58 marker was overexpressed on $CD10^{+}$, $CD34^{+}$ cells at diagnosis and MRD detection, in cases with detected

hematogones intensity of CD58 expression was lower. It appears, that in certain instances CD58 is able to distinguish leukemic and normal progenitor cells, as it was published by others [5].

Multiparametric flow cytometry is the method for precise single-cell definition of pathological leukemic cells at diagnosis and provides relevant information regarding the assessment of MRD in patients in clinical remission.

The authors gratefully acknowledge the physicians from the Department of Pediatric Oncology of University Children's Hospital and from the National Cancer Institute, Bratislava, Slovakia, for patients' samples and referrals. The technical cooperation of Mrs. L. Ševulová and Mrs. A. Kovaríková is greatly appreciated.

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