The knowledge on the '3rd type hematogones' could contribute to more precise detection of small numbers of precursor B-acute lymphoblastic leukemia*

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Bone marrow hematogones (benign B-lymphocyte precursors) may cause diagnostic problems due to their morphologic and immunophenotypic similarities with neoplastic lymphoblasts. Hematogone populations in presented study containing 358 bone marrow specimens of 251 individuals always exhibited a continuous and complete maturation spectrum of antigen expression typical for normal evolution of B-lineage precursors; lacking aberrant or asynchronous antigen expression. In contrast lymphoblasts of 19 bone marrows of precursors B-ALL patients showed maturation arrest and exhibited several immunophenotypic aberrancies. Hematogones were identified by 4-color flow cytometry using optimal antibody combinations in many bone marrow samples. They were more commonly found in higher numbers in children, and there was found a general decline in hematogones with increasing age. Bone marrow hematogones were separately assessed as hematogones 1 population of early stage and hematogones 2 of mid-stage precursor B-cells, respectively. In some (about 30%) of hematogones a third type hematogones could be assessed in bone marrow samples. This small B-cell subpopulation was defined by CD10-positivity, coexpressing more mature markers CD19,CD20,CD22 and CD45^{bright}. These cells obviously blended with those of mature B-lymphocytes (CD10-negative) on CD45/SSC, and could be better recognized on CD10-gating. Quantitative immunophenotyping of this study completed the percent antigen expression data in two main hematogone subtypes and lymphocytes in 16 bone marrow specimens and precursor B-ALL lymphoblasts in some samples.

Increased information on benign B-lymphocyte precursors, especially that of existence of 'the 3rd type hematogones' could provide a basis for better discrimination of B-leukemia cells even in a very small amounts.

Key words: hematogones, lymphoblasts, normal bone marrow, flow cytometry, multiparameter immunophenotyping, expression profile

Multiparameter flow cytometric (FC) analysis of bone marrow (BM) samples is performed for identification, frequency assessment, and more detailed definition of various leukocyte subpopulations when abnormal hematopoiesis is suspected. The BM compartment contains cells of different hematopoietic lineages with various maturation stages per lineage. In healthy individuals, the BM precursor cells guarantee continuous production of the various hematopoietic differentiation lineages. Especially in the B-cell lineage, multiple stages have been defined based on their immunophenotype [5, 8, 14]. The differentiation and maturation can be monitored by changes in cytomorphology and immunophenotype. Knowledge of the expression levels of various lineage-specific, mature and immature markers in normal hematopoietic development provides a basis for recognition of abnormal differentiation patterns [3, 16].

Various studies have reported the morphology, immunophenotype and frequency of benign B-cell precursors known as hematogones, in bone marrow of children and adults with a variety of benign and malignant conditions [6, 9, 10, 13]. Hematogone populations consist primarily of mid-stage B-cells with a lower proportion of early-stage cells and some mature B-cells. In common usage, the early and mid-stage B-cells are called hematogones. Bone marrow hematogones may cause diagnostic problems for their morphologic and immunophenotypic similarities with neoplastic lymphoblasts [16, 17].

The purposes of this multiparametric flow cytometry study were: 1) define exactly hematogones with the most appropri-

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ate antibodies and new technical approaches, 2) quantify hematogones (separately those with early stage and mid-stage B-cells) across age groups, 3) to extend their characteristics for molecule numbers (molecular equivalent of soluble fluorochrome – MESF values) of individual markers present, and 4) to compare their immunophenotype with that of neoplastic lymphoblasts, mainly B-cell precursor ALL.

Patients, material and methods

In this paper we sought to study in more details the BM hematogone characteristics in children and adults in unselected series of 358 consecutive BM samples submitted for flow cytometric (FC) examination to our laboratory and to provide criteria for the exact discrimination between neoplastic lymphoblasts of B-cell precursors ALL and benign hematogones.

Patients and specimens. A prospective 4-color FC of hematogones was performed during 14-month period. During this time 358 bone marrow specimens (152 from children and 206 from adults) of 251 individuals (132 males and 119 females; age range 1 month – 83 years) in different age categories were assessed in more details for hematogone characteristics in children and adults in unselected series. A total of 31 BM specimens from 28 patients (mean age 26.4) with newly diagnosed or relapsed B-ALL were analyzed during the time period of the study. 19 specimens were diagnosed as precursor B-ALL (mean age 29.0; 6 males and 13 females), 3 as pro-B ALL (CD10-) (median age 30.6; 2 males and 1 female), 4 as pre-B-ALL (cytIgM+) (mean age 18; 4 males) and 2 patients had mature B-cell ALL (sIg+) (mean age 12; 1 male and 1 female) phenotype. In two more patients were in BM simultaneously discovered hematogone and residual precursor B-ALL populations (both involved in previous categories). Cells of all cases B-ALL were immunophenotyped and BM phenotypes in precursor B-ALL were compared with those of hematogones.

Heparinized BM samples of patients at initial diagnosis or during follow-up for suspect hematopoietic disease were studied. Multiparameter analysis of lymphocytes was performed using a lysed-whole-blood technique with commercially available red cell lysing solution (Optilyse B, Immunotech, France). Percentages of cell types were determined based on total events of all BM cells. The cell labeling and membrane fixation/permeabilization procedures have been described in detail previously [2, 11].

Flow cytometric analysis. All antibodies directly conjugated to 4 different fluorochromes to specifically define B-cell precursors, B-lymphocytes and leukemia cells of B-lineage were purchased mainly from Immunotech, Marseille, France, except for some from Caltag, Ca, USA. Three-, four-combinations of monoclonal antibodies were used in standardized protocols:
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-F/CD10-PE/CD45-PC5. Some other combinations of monoclonal antibodies were used to detect aberrant phenotypes in leukemia blasts. Measurements of antibody labeling were performed by multiparameter FC analysis with EPICS ALTRA flow cytometer equipped by EXPO 32 analysis program (both from Beckman Coulter). The background fluorescence level for each specimen was established using cells incubated with the appropriate fluorochrome-coupled isotype control. At least 30 000 cells were evaluated per sample and percentages of cell subtypes were determined based on total events. Immunophenotypic abnormalities in leukemia blasts were determined on the basis of deviation from normal patterns of B-cell development.

Evaluation of marker density. For evaluation of antigen molecules, expressed by mean fluorescence intensity, FC was calibrated by fluorescent calibration microbeads. Coulter (Immunobrite, Coulter Corporation, Hialeah, FL, USA) calibration particules with one blank and four different fluorescence intensities relevant to molecules of equivalent soluble fluorochrome (MESF) were used. Fluorescence histogram of these particles was measured on logaritmic scale and linear regression was calculated.

Statistical analysis. The results of the study were expressed as mean, median and standard deviation. Associations of percentage of bone marrow hematogones with age were analyzed using Spearman rank correlation coefficient. Statistical analysis of differences between two parameters was evaluated with the Mann-Whitney U test. A p-value less than .05 was considered statistically significant.

Results

Bone marrow hematogones by patient age. From a total of 358 consecutive BM specimens analyzed hematogones were identified in 166 BM samples (46.36%). They were more commonly found in higher numbers in children and a significant general decline in hematogones with increasing age was found. The scatter plots in Figure 1 a,b,c show the percentage of total hematogones and separately those of early and mid-stage hematogones in relation to patient age. In the followed cohort, the mean % of total values of hematogones is 2.53, of early hematogones (Hg 1) 0.407 and those of mid-stage hematogones (Hg 2) 2.13. There was a significant decrease in a total and mid-stage hematogone (Hg 2) values with increasing patient age as shown by the regression line; the values were less numerous in early hematogones (Hg 1) with the same tendency. A total percent of hematogones with 5% or more of the total BM events in 61 (17.03%) was found (Fig. 2). The difference between values of ≥5% hematogones of samples from patients aged ≤16 years – 6.74%, and from those of ≥16 years – 1.77%, was found to be statistically significant ($p<.0001$), Figure 3.

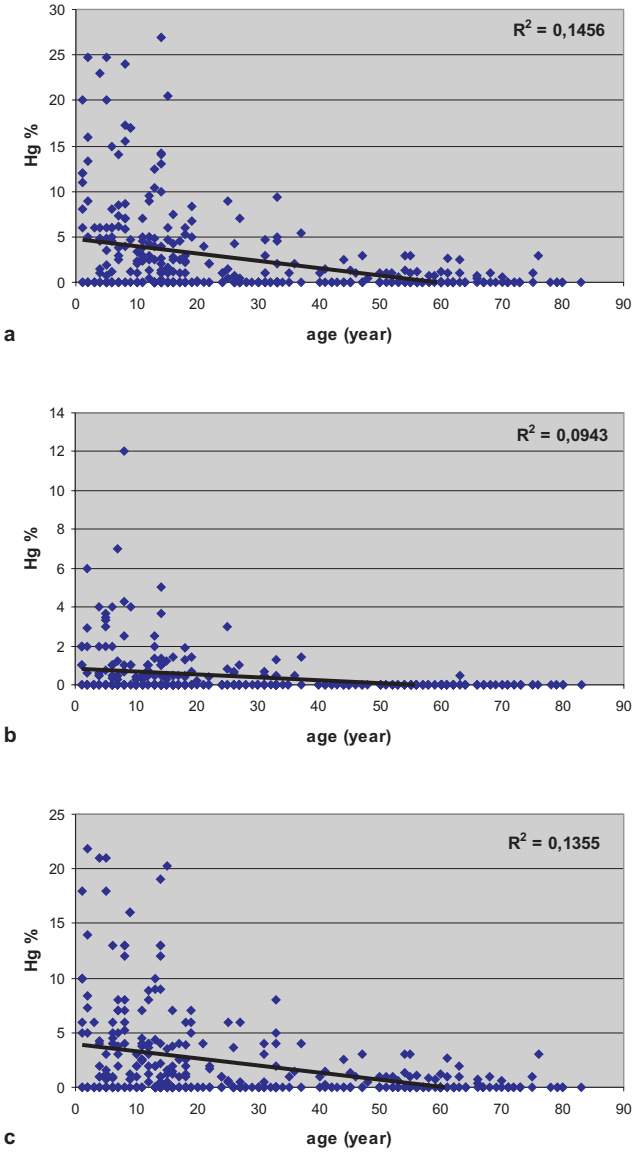

Figure 1. The scatter plots relating percent bone marrow total (a), early (b) and mid-stage (c) hematogones to patient age for all 358 specimens. There is a significant decrease in the pecent hematogones with increasig age as shown by the regression lines. One case in Figure 1 a and b was off the scale and it is not shown – a 14-year-old child with 42.7% hematogones.

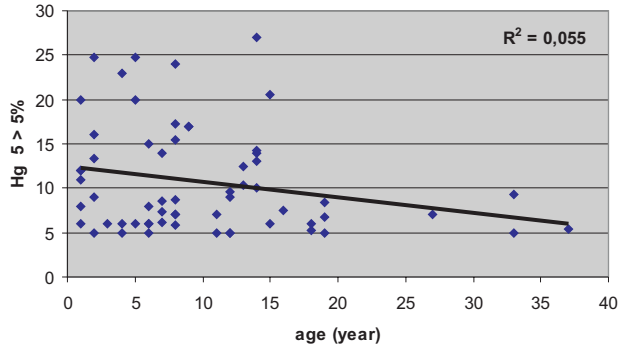

Figure 2. A scatter plot given the total percent of bone marrows with 5 or more hematogones, so-called "increased hematogones", in 61 specimens (17.03%). There is the similar significant decrease in the percent hematogones with increasing age for the total cohort of the 358 specimens. One case was off the scale and it is not shown – a 14-year-old child with 42.7% hematogones.

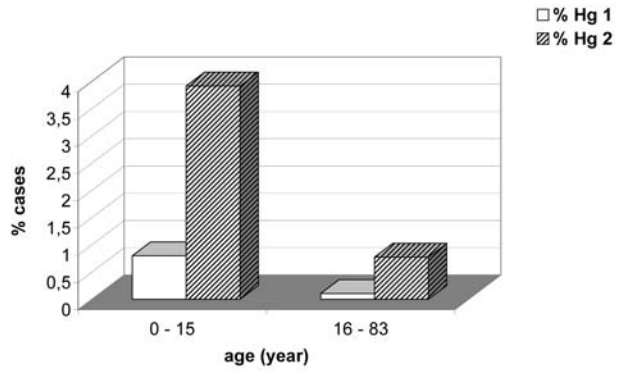

Figure 3. The percent of cases with less then 16 and more then 16 years in BM specimens for early (Hg 1) and mid stage (Hg 2) hematogones. The difference was found to be statistically significant (<.001).

Immunophenotypic characteristics. Hematogones were characterized using well-established criteria, including very low side scatter values, variable expression of CD45 and bright expression of CD19,CD10,CD38. The lowest percentage of hematogone values detected in our study was 0.17 and the highest one 42.7. A minor subpopulation expressed the early stage cell markers CD34 or TdT and lacked the mature antigen CD20 (hematogones 1). The mature B-cell marker CD20 dominated in mid-stage B-cells (hematogones 2); both types of hematogones showed a complex spectrum of antigen expression that defines the normal antigen evaluation of B-cell precursors and they lacked the aberrant expressions. An example of maturation spectrum is given in Figure 4. In some (about 30%) BM specimens with hematogones, the presence of very small amounts (mean 2.15; range 0.7–4.5) of further subpopulation of hematogones (named here as hematogones 3) could be assessed. This small cell subpopulation – 'the 3rd type hematogones' is defined by CD10-positivity, coexpressing more mature markers CD19+,CD20+,CD22+ and CD45^{bright}. These cells on CD45/SSC dot plot obviously blended with those of mature lymphocytes (CD10-). This subpopulation could be better recognized on CD10 gating. An example of these 3 benign precursor B-cell subpopulations is shown in Figure 5.

The BM lymphoblasts in all 19 patients with precursor B-ALL, in contrast to hematogones, exhibited incomplete

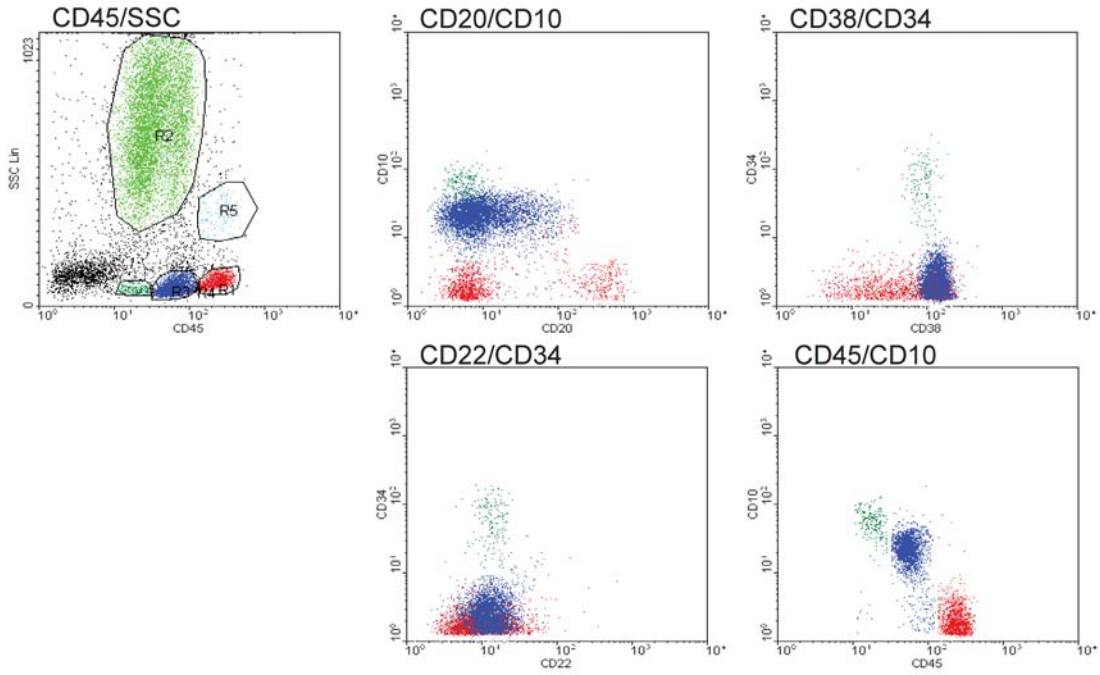

Figure 4. A continuous staining pattern in a CD10/CD20 dot plot as a result of stepwise loss of CD10 and the gradual gain of CD20 during maturation was found to be the most important marker of normal B-cell differentiation stages. Dark-green indicates early hematogones (1.4%), blue mid-stage hematogones (28%) and red ones mature lymphocytes (8%) of all BM analyzed cells.

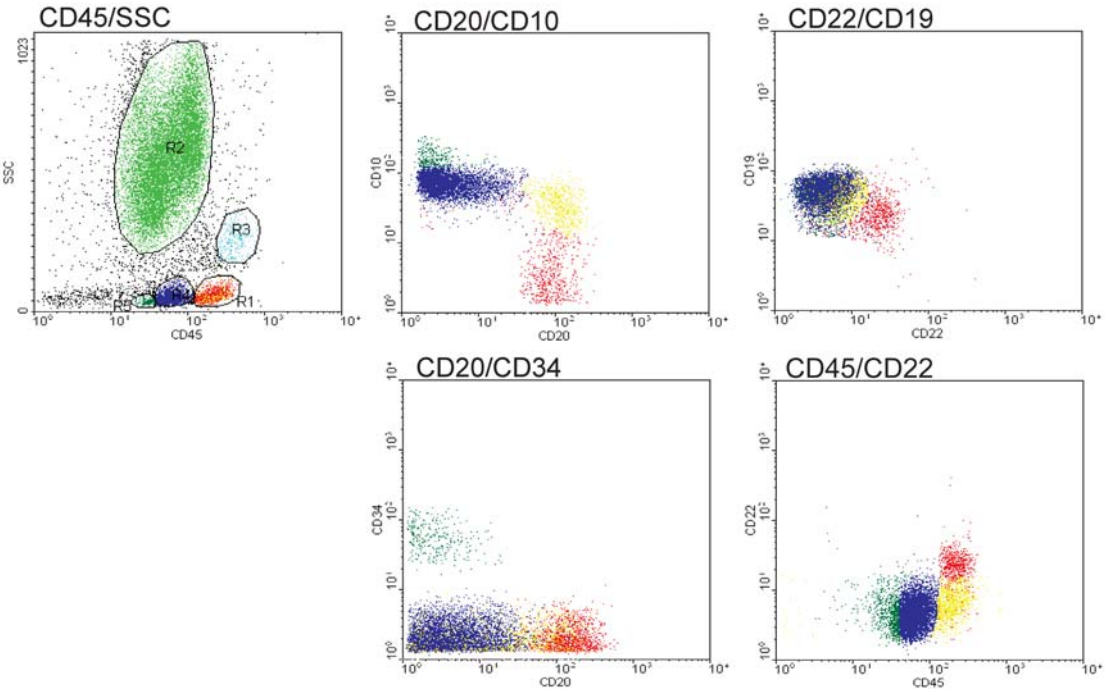

Figure 5. Flow cytometry histograms of a BM specimen with three types of benign B-lymphocyte precursors and mature lymphocytes. Dot plots illustrate the normal pattern of B-lymphocytogenesis with the 4-color antibody combination. Dark-green are early, dark blue mid-stage hematogones, yellow immature lymphocytes ('the 3rd type hematogones') and red mature lymphocytes. Light-blue are monocytes and light-green granulocytes. Immature lymphocytes (yellow) on CD45/SSC dot plot usually blend with mature B-cells. BM cells accounted for hematogones 1 and 2, 2% and 18%, respectively, for lymphocytes 15%, and for neutrophils and monocytes 62 and 1%, respectively. 15% of lymphocyte population formed 2.2% of the 3rd hematogones, 3% mature B-lymphocytes and the rest (9.8%) T-lymphocytes.

maturation and immunophenotypic asynchrony and aberrancy that deviated from the complex spectrum of antigen expression of normal stages of maturation (Fig. 6). The lymphoblasts from all 19 patients were studied with complete panels of antibodies and exhibited at least 2 aberrancies. In 2 BM specimens a rarely observed simultaneous expression of both, lymphoblasts and hematogones in a patient with progenitor B-ALL was followed. The example is given in Figure 7.

MESF values of some B-cell antigens. MESF values showed to be another marker characterizing early, mid-stage hematogones and lymphocytes and some markers in precursor B-ALL (Fig. 8). It could be concluded that high percent expression of CD10 in early and mid-stage hematogones comparing to lymphocytes is accompanied by more molecules of CD10, too. Similarly, CD34 expressed significantly more molecules in early hematogones (Hg 1) comparing to mid-stage hematogones (Hg 2) and lymphocytes. Similarly, significantly more molecules of CD45 were present in lymphocytes comparing to those of the other two types of BM subpopulations in the specimen. In individual precursor B-ALLs, the expressed molecule numbers (MESF values) were often different for some of the same antigens. Last picture of Figure 8 gives an example showing low % value of CD45 (only MESF values shown in Figure 8) followed by its fewer molecule numbers. In opposite, in the same patient CD34 expressed fewer molecules but % expression of this antigen was very high (only MESF values shown in Figure 8).

Discussion

In the prospective multiparameter study during the time-period of 14 months a total of 358 BM specimens were examined. FC study confirmed age-related shifts in normal BM development and extended it for separate values of early, mid-stage (Fig. 1 a,b,c) and newly defined immature B-cells, 'the type 3 of hematogones' (Fig. 6). This 'type hematogones' seems to be the analogy of VAN LOCHEM's cell type described in normal B-cell development as immature B-cells in chilhood BM of 4-year old healthy child [14].

Recent comprehensive study of McKENNA et al [9] surveyed the incidence of a total hematogones in an unselected series of 662 consecutive BM samples submitted for FC also at a single centre. The investigators reported that in infants <2 years of age total hematogones averaged 9%, in 2–5 years the percent decreased to 3.9 and in persons >50 years of age the average was <1%. In our study the averages for hematogones were smaller that those of the McKENNAS', as a result of using in our study the erythrocyte lysis technique for samples processing instead of gradient separation. The highest yet described percentages of hematogones in any age group, averaging 34% of all cells, were reported by RIMSZA et al [12] in their preterm neonates samples study. Furthermore, most prior studies on the subject have been performed on relatively small patient cohorts, but even these studies supported our findings on age distribution of hematogones [8, 12].

Hematogones are identified by 4-color FC analysis using optimal antibody combinations in many bone marrow samples. It follows from our study and as well as from others [9, 14, 15] that B-lymphocytogenesis is a phenomenon of marrow regeneration. It appears that this propensity for increased values of hematogones stimulated by certain disease states diminishes with aging (Fig. 1, Fig. 3). Increased hematogones are observed most commonly in patients with lymphomas, in BM regenerating states following chemotherapy or BM transplantation and in different cytopenias [10, 13]. The results are in agreement with data provided by our clinical coworkers. Hematogones are only rarely detected in peripheral blood other than in minute numbers [4].

Bone marrow hematogones (benign B-lymphocyte precursors) may cause diagnostic problems for their morphologic and immunophenotypic similarities to neoplastic lymphoblasts. Especially arbitrarily designates 5% as "increased" hematogones in our study and in the study of McKENNA et al [10] are more likely to be confused with neoplastic lymphoblasts.

In the present study hematogone populations always exhibited a continuous and complete maturation spectrum of antigen expression typical for the normal evolution of B-lineage precursors and they lacked aberrant or asynchronous antigen expression (Fig. 4, 5). In contrast, lymphoblasts showed maturation arrest and exhibited several immunophenotypic aberrancies, shown in Figure 6, 7. In most cases of acute leukemia, especially those of precursor B-ALL, we found the aberrant immunophenotype often in a positions of the malignant cell population at a separate place of the dot plot template; the cells are located in a so-called 'empty space' where normally no healthy cells are found [7, 11]. Thus, the lymphoblasts in ALL form small clusters of events falling also outside the normal template for hematogones.

Some authors have also reported methods to discriminate between normal B-cell precursors and neoplastic lymphoblasts. FARAHAT et al found B-lineage ALL lymphoblasts to express fewer TdT and CD19 and more CD10 molecules than did hematogones [1]. In our study the method of quantitative analysis was also used to show differences among different antigen values expressed in different B-lymphocyte subpopulations (early and mid-stage hematogones and lymphocytes) and precursor B-ALL lymphoblasts.

Our findings in correlation with those of other investigators suggest that multiparameter flow cytometry is able to reliably distinquish hematogones from residual ALL in virtually all cases when optimal antibody combinations are used. The possibility to recognize in BM very small amounts (mean 2.15) of further subpopulation of hematogones (named here as hematogones 3) could substantially improve the detection possibilities of residual blasts in B-acute leukemia.

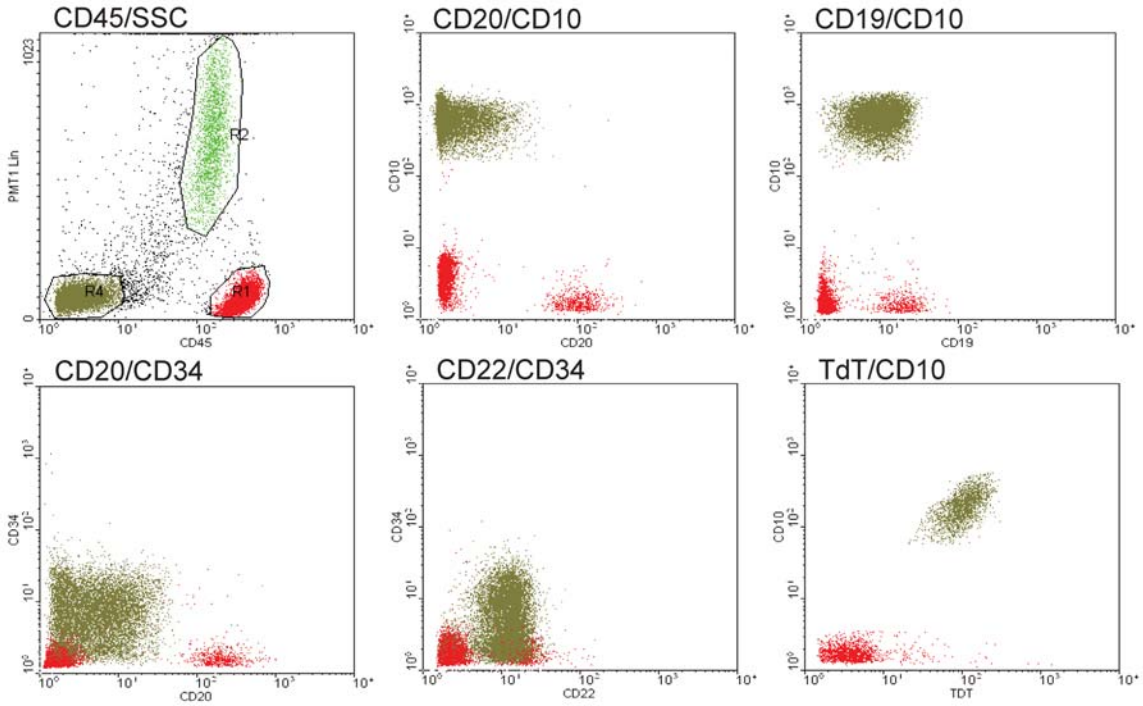

Figure 6. Flow cytometry histograms in a BM specimen of a patient with precursor B-ALL at diagnosis. Dot plot illustrate on CD45/SSC lymphoblasts (khaki, 60%) and residual lymphocytes (red, 22%) and neutrophils (13%). Lymphoblasts exhibit abnormal distribution comparing to normal lymphocyte hematopoiesis (abnormal CD34/CD20 expression, over-expression of CD10, under-expression/negativity of CD45 and nuclear TdT-positivity).

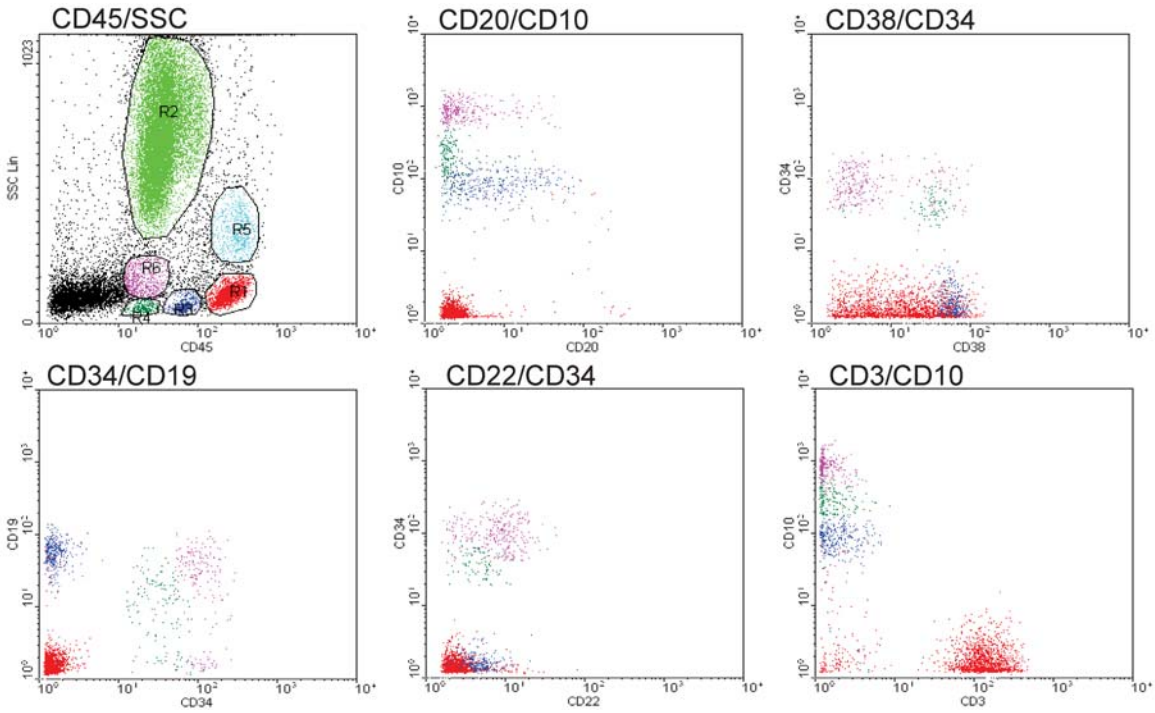

Figure 7. Four-color dot plot analysis of BM specimen with simultaneously present 2 different stages of hematogones (dark-green and blue color, respectively) and small number of minimal residual lymphoblasts (violet). Mature lymphocytes are red (10%), light-blue monocytes (2.3%) and light-green granulocytes (72%). Bone marrow lymphocytes accounted for early hematogones 1%, for those of mid-stage hematogones 2.23%, for pathological gate 1.5% and for mature lymphocytes 10%.

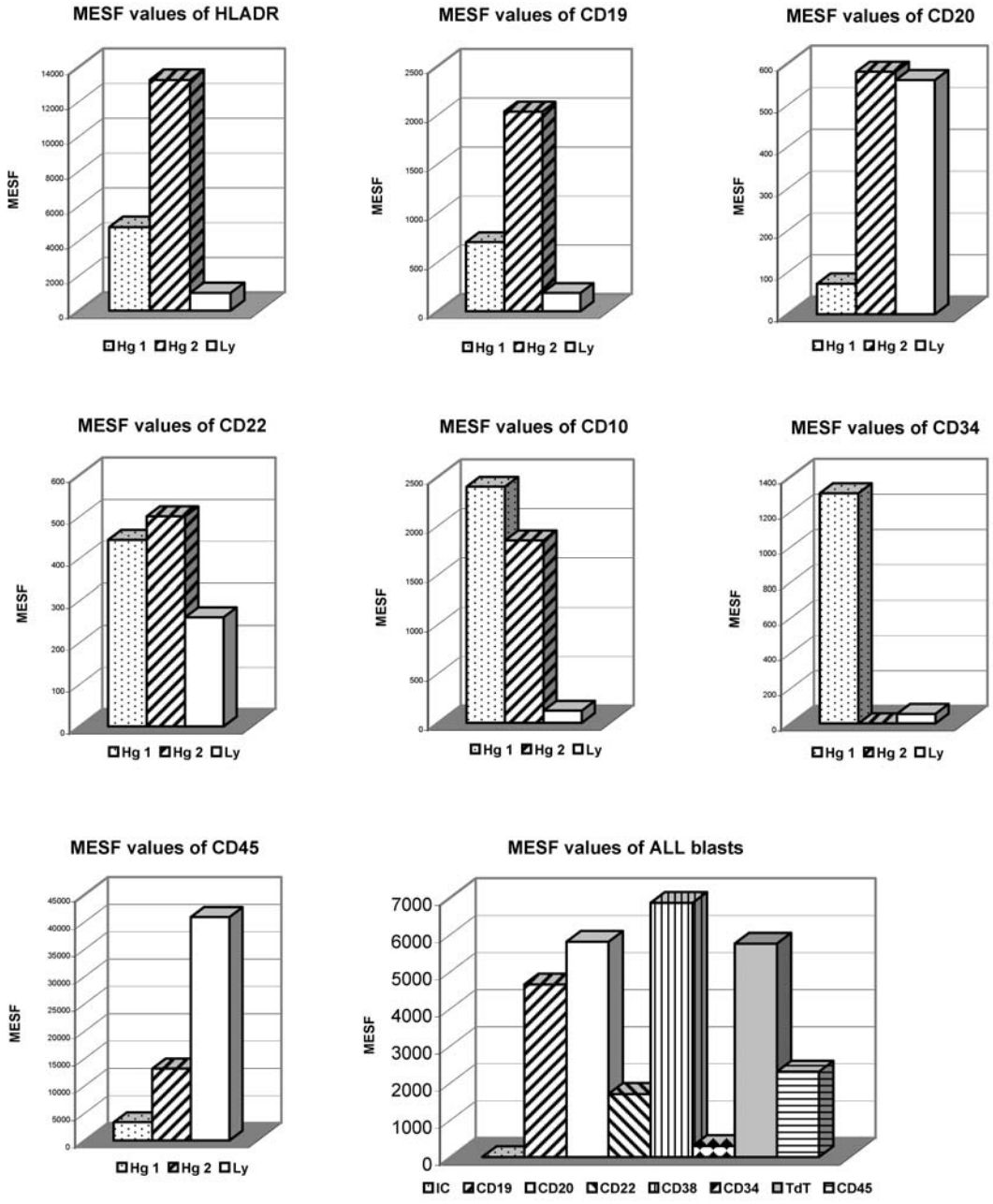

Figure 8. Molecule equivalent of soluble fluorochrome (MESF) values were compared in early (Hg 1) and mid-stage (Hg 2) hematogones and mature lymphocytes of 16 BM specimens and some precursor B-ALL patients for 7 markers used for B-cell definition, i. a. HLA-DR, CD19, CD20, CD22, CD10, CD34 or nuclear TdT, and CD45.

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