Normal maturation sequence of immunoglobulin light and heavy chains in hematogone stages 1, 2 and 3 in acute leukemia after treatment

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The cellular diversity of bone marrow samples was studied by using multi-dimensional cluster analysis of six-parametric flow cytometry data (four CD, forward scatter and side scatter), focusing mainly on acute leukemia blast cells and regeneration of normal B-cells, hematogones. This approach should enhance the ability to study normal hematopoiesis, and to identify and monitor hematopoietic disorders. The study was performed on a homogeneous group of patients (mainly children), all of them after finishing complete therapy for AL, mostly B-ALL. In all of these patients complete pattern of all three individual Hg stages was present. Maturation spectra of surface immunoglobulin kappa (sIgκ) and lambda (sIgλ) light chains and IgM, IgA heavy chains in all three stages of Hgs are presented as reliable reports on sIgs as their incidence on Hgs are scarse and even contradictory. The Ig expression paralles CD20 expression. SIg of light (κ,λ) and heavy (IgM, IgA) chains were completally absent in stage 1 Hgs and their expression increased through stage 2 to 3; IgM was expressed similarly. Light Ig chains κ/λ were expressed in a polytypic way. The results completed information on normal maturation sequence of bone marrow stage 1, 2 and 3 hematogone regeneration in treated acute leukemia patients.

Key words: Acute leukemia, benign B-lymphocyte precursors, hematogones, multiparameter flow cytometry, surface Ig of light (κ,λ) and heavy (IgM, IgA) chains maturation sequence

Multiparameter flow cytometry with optimally selected antibody combinations has expanded the use of this technique beyond traditional applications in oncohematology. By analyzing quantitative patterns of antigen expression on discrete populations or „clusters", one can detect immunophenotypic aberrancy in specific cell populations in relation to normal populations. Evaluation of patterns of antigen expression can also be used to supplement conventional methods in the diagnosis and subclassification of certain types of hematologic neoplasia. Finally, the diagnosis of regeneration/reactive populations present in hematolymphoid system can be facilitated by the detection of immunophenotypic characteristics. Examples of application of multiparameter flow cytometry with particular relevance to benign B-cell precursors, hematogones (Hgs) were recently discussed [1, 2, 3].

In our previous study [4] we discriminated acute leukemia (AL) cells and Hgs over the disease course from diagnosis to remission and relapse. We followed Hgs in the course of bone marrow (BM) regeneration in a total of 669 bone marrow aspirates in leukemia patients with B-cell ALL (B-ALL), acute myeloid leukemia (AML) and T-cell ALL (T-ALL). The three individual physiological maturation phases of B-lymphocytes (hematogone stage 1, 2 and 3) were studied by four-color flow cytometry in the course of bone marrow regeneration in leukemia patients. Multiple stages of hematogones were observed twice as frequently in B-ALL (73,8%) and T-ALL (69,2%) samples as in AML aspirates (34,1%). Stage 3 hematogones matching the Van Lochem´s cell type of normal immature B-cells [1, 3] were found usually in children and were thus frequent in B-ALL. Hematogones showed extremely high phenotypic stability unaffected by disease or therapy or their coincidence with leukemia cells.

Presented study represents a homogeneous group of patients, mainly children, majority of them were after finishing complete therapy for AL, mostly B-ALL. Complete pattern of all three individual Hg stages was present in all these patients. We studied the maturation spectra of sIgκ and sIgλ light chains and IgM, IgA heavy chains in all three stages of Hgs. Precise reports on sIgs and their incidence on Hgs are scarse and contradictory [2, 5, 6, 7]; majority of these results

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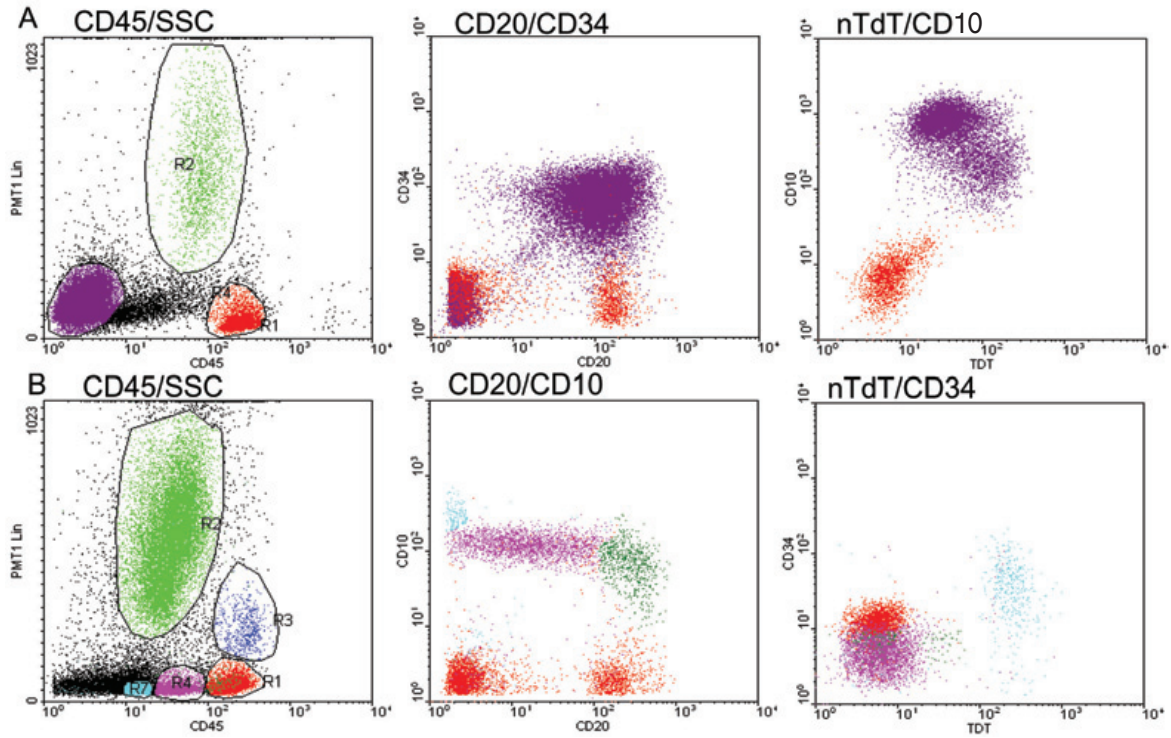

Figure 1 A Bone marrow flow cytometry histograms from a 16-year-old female with precursor B-ALL at diagnosis. Dot plots illustrate on CD45/SSC lymphoblasts (dark violet) 78%, residual lymphocytes (red) 14% and granulocytes (light green) 8%. Lymphoblasts exhibited abnormal distribution pattern comparing to normal residual lymphocytes (down-regulation/negativity of CD45, asynchronous coexpression of CD20/CD34 and expression of nTdT-positivity and CD34 expression).
B Flow cytometry histograms of a bone marrow (BM) specimen in a 6 year-old boy with precursor B-ALL in remission with three types of benign B-lymphocyte precursors. 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. BM cells accounted for hematogone (Hg) stage 1 (turquoise) 2% and stage 2 (violet) 18%, for lymphocytes (red) 15%, and for neutrophils (light green) 62%, and monocytes (dark blue) 1%. 15% of lymphocyte population consisted of the stage 3 Hg (2.2%, dark green), mature B-cells (3%) and T-lymphocytes (9.8%). Granulocytes and monocytes are omitted in further pictures.

were obtained by immunohistochemical stains enabling to compare them with our results obtained by flow cytometry.

Material and methods

Patients. Using the multiparameter 4-color flow cytometric analyses from a total of 25 BM aspirates of both, children and adults, were evaluated. Samples were acquired after therapy from 20 patients with B-ALL, from 1 patient with AML, and from 4 patients with T-ALL. In all these patients complete pattern of all three individual Hg stages was present. The mean age±SDM in B-ALL patients was 10.8±8.1 (20 patients, from 3 to 25 years), for AML patients the value was 38 (1 patient 38 years); and for T-ALL patients the values were 21.8±16.2 (4 patients, from 1 to 36 years). Children were treated according to the BFM (Berlin-Frankfurt-Münster) protocols (ALL IC BFN 2002, AML-BFM 98) and adults according to GMALL (German Multicenter ALL) for ALL. AML adult patients were treated according to standard protocols (combination of antracycline and cytarabine/3+7).

Immunophenotyping of BM cells for leukemia and benign B-cell precursors was performed using a lysed-whole-blood technique.

Flow cytometry for hematogone identification. Combinations of three to four fluorochrome-conjugated antibodies (FITC – fluorescein isothiocyanate, PE – phycoerythrin, ECD – R-phycoerythrin-texas red and PC5 – phycoerythrin-cyanin 5) were routinely used to specifically define Hg, B-lymphocytes, and leukemia cells of B-, T- and myeloid-lineage. All monoclonal antibodies directly conjugated by fluorochromes were purchased from Immunotech (Marseille, France). Specific combinations of CD20-FITC/CD10-PE/CD45-ECD/CD19-PC5, CD22-F/CD34-PE/CD45-ECD/CD19-PC5 and nTdT-F/CD10-PE (CD34-PE)/CD45-PC5 were used according to standardized protocols for B-lymphocytes. To detect surface immunoglobulin kappa (sIgκ) and lambda (sIgλ) light and IgM and IgA heavy chains their combination with CD10,

CD34 and CD20 was used. Other combinations of monoclonal antibodies, as described earlier [8, 9], were used to detect AML, T-ALL and aberrant phenotypes in leukemia blasts. A minimum of 30 000 cells were evaluated per sample and percentages of cell subtypes were determined.

Results

In the study multiparameter flow cytometric analysis was used to discriminate leukemia cells and benign B-lymphocytes (hematogones). Precise definition of leukemia populations was based on the identification of leukemia-associated phenotypes (LAPs), which generally result from marker expression of another lineage, maturation-asynchronous antigen expression, over- or under-expresion of normal antigens, and abnormal light-scatter patterns [2, 10]. LAPs are present in the majority patients with AL and could be defined even if leukemic cells were present in minute amouts [4]. The BM lymphoblasts and myeloblasts showed incomplete maturation and immunophenotypic deviations from the normal antigens spectrum during B-cell maturation. An example is given in Figure 1 A.

In opposite, Hgs varied in their antigens expression ranging from B-cell precursors to mature B-lymphocytes and regularly lacked aberrant marker expression. By multiparameter analysis 3 different stages of Hgs could be easily recognized – Hg 1, 2 and 3 (Figure 1 B). Stage 1 Hgs expressed the early stage cell markers CD34 and nuclear terminal deoxynucleotidyl transferase (nTdT) and lacked the more mature B-cell marker CD20, whereas stage 2 Hgs was characterized by progressive up-regulation of CD20. Stage 3 Hgs was defined by CD20 expression to a similar level as seen in mature B-lymphocytes, by CD10 positivity and $CD45^{bright}$. On CD45/SSC scatter plots stage 3 Hgs (CD10+) were mixed with mature B-lymphocytes (CD10-). Table 1 summarizes the key antigen markers to define the three stages of hematogones.

We investigated the expression of surface Igκ and Igλ light and IgM, IgA heavy chains in cases with a complete pattern of all three Hg stages. The appearance of Ig was associated with the expression of CD20. Stage 1 Hgs was entirely negative for both sIgκ/λ and IgM, and for CD20. Stages of 2 and 3 Hgs that were positive for CD20 expressed variable both types of sIg light chains (polytypic expression) and IgM/IgA heavy chains. IgM/CD10 scatter plots in stage 2 Hgs show the IgM positivity expressed in two patterns, lower fluorescence intensity ($IgM^{-/dim}$) and higher fluorescence intensity (IgM^{bright}). Similar pattern of fluorescence intensity parallers the CD20 expression profile in stage 2 Hgs. Figure 2 shows an example of the maturation sequence for sIg light chains (κ and λ) and heavy chains (IgM and IgA) in all three Hg stages. In one BM aspirate the expression of Hg sIgs was present in B-ALL patient in coexistence with small pathological gate (MRD) (Figure 3). The coexistence of leukemia cells did not hamper the identification of Hg stages.

The data for individual mean percent of Hg stages 1, 2, and 3 of total bone marrow events in AL patients in clinical remission after therapy are shown in Table 2. Cells in stage 1 Hgs constituted a significant minority and cells in stage 2 formed a significant majority in Hg cells unrelated to leukemia type (B-ALL, AML and T-ALL).

Discussion

The study of normal and malignant hematopoiesis requires the analysis of heterogeneous cell populations using multiple morphological and molecular criteria. Flow cytometry has the capacity to acquire multiparameter information of hematopoietic cell populations, utilizing various combinations of >200 molecular markers (cluster of differentiation, CD) [10, 11]. Current multiparameter FC analyses are based on serial gating of two-parametric scatter plots. Multiparameter immunophenotyping [1, 11, 12] of BM aspirates enables evaluation of leukemia therapy effectivenes by sensitive detection of malignant cells and thus detection of MRD (13, 14, 15,).

Early TdT-positive B-cell precursors have been characterized extensively in bone marrow, where they have long been recognized morphologically as benign immature lymphoid cells, or so-called hematogones [16]. Numerous studies have described comprehensively the morphologic [24] and immunophenotypic characteristics of these cells (18, 19, 20, 21) and their differential diagnosis from acute lymphoblastic leukemia [2, 3]. These studies established that TdT-positive lymphoid cells in bone marrow are B-cell precursors showing a range of maturation [1, 3], increase numerically in a reactive conditions [2], and are most nu-

Table 1. Normal maturational sequence of bone marrow stage 1, 2 and 3 hematogones

Hematogones				Mature B cells
Stage 1	**Stage 2**		**Stage 3**	
TdT				
CD34				
$CD45^{dim}$	CD45	CD45	$CD45^{bright}$	$CD45^{bright}$
$CD10^{bright}$	CD10	CD10	CD10	**CD10negat**
CD19	CD19	CD19	CD19	CD19
$CD22^{dim}$	$CD22^{dim}$	$CD22^{dim}$	$CD22^{dim}$	CD22
$CD38^{bright}$	$CD38^{bright}$	$CD38^{bright}$	$CD38^{bright}$	$CD38^{bright/-}$
		CD20	CD20	**CD20**
	sIgκλ, IgM	sIgκλ, IgM	sIgκλ, IgM	sIgκλ, IgM

Table 2. Cells in different Hgs maturation stages of acute leukemia patients in remission after therapy (%)

		Hematogones		
Acute leukemia type	**No**	**stage 1**	**stage 2**	**stage 3**
B-ALL	20	1.4±1.1	8.7±5.6	1.5±1.7
AML	1	0.4	5.2	2.0
T-ALL	4	0.6±0.2	3.6±2.1	0.7±0.5
total	25	**2.4**	**17.5**	**4.2**

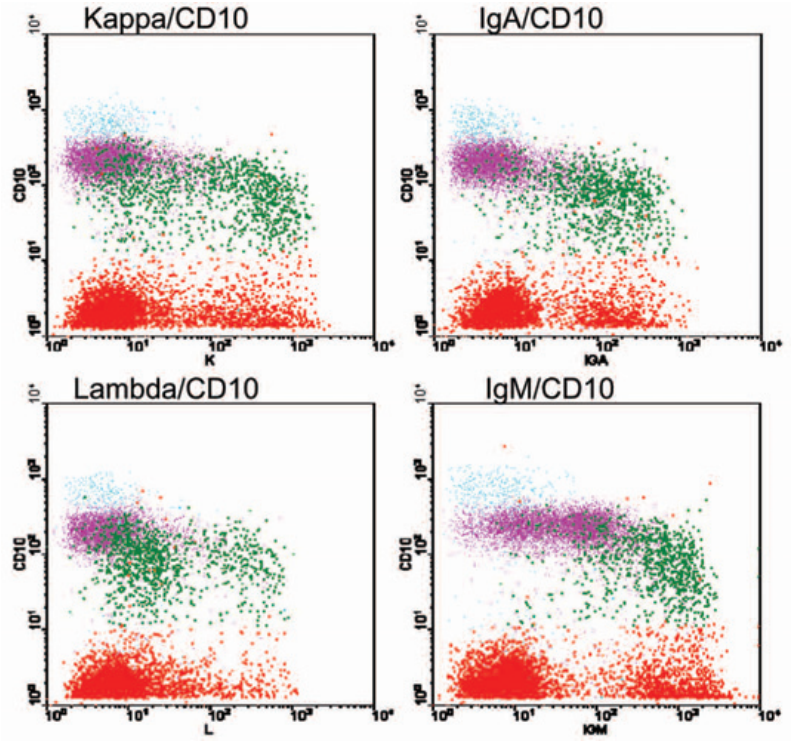

Figure 2 The example of maturation sequence of kappa and lambda light and IgM and IgA heavy chains surface immunoglobulins in stage 1 (turquoise), stage 2 (violet) and stage 3 hematogones (dark green). Mature bone marrow lymphocytes (red) are shown for comparison (majority of them form T-lymphocytes).

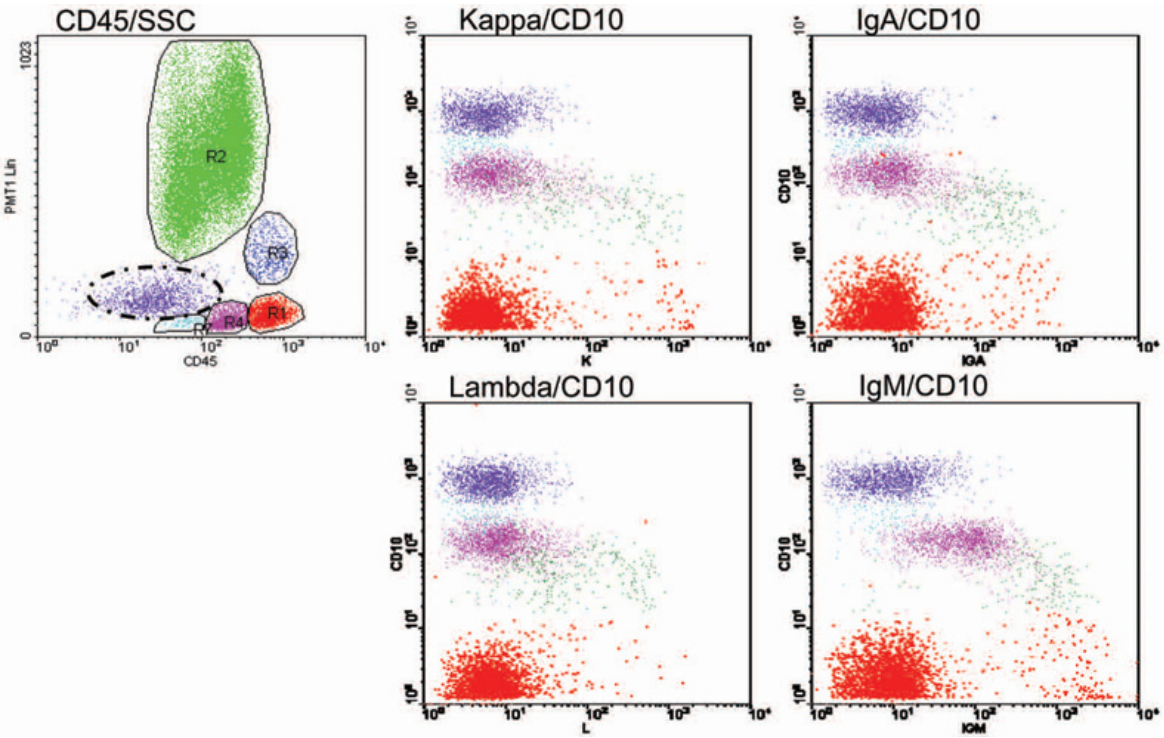

Figure 3 The example of maturation sequence of kappa and lambda light and IgM and IgA heavy chains surface immunoglobulins in stage 1 (turquoise), stage 2 (violet) and stage 3 hematogones (dark green). In This BM aspirate the expression of Hg sIgs was in B-ALL patient present in coexistence with small pathological gate (dot-and-dashed line).

merous in infants and children [2, 3, 17]. In addition, the majority of patients with Hgs had a history of concurent or past malignant neoplasms [20, 21] and depends on preceding therapy [22, 23].

McKenna et al. [5, 24] presented a comprehensive study of total Hgs in an unselected series of consecutive BM samples of a single centre. They reported that in infants less than two years of age Hg incidence was 9%, in 2- to 5-year-olds it was 3.9% and in subjects older than 50 years of age Hg were found in less than one pecent. Similar results on large patients´ cohort were found in our studies [3, 4], in which Hgs were analysed in different maturation stages. Studies performed on smaller patient cohort have also shown a similar age distribution [18, 19]. Rimsza et al. [25] reported hematogones to represent 34% of cells in preterm infants´ or neonates´ BM samples. They used a panel of immunohistochemical stains to characterize early and mid-stage Hgs in 11 neonates and two additional cases by flow cytometry.

In this and in previous studies [3, 4] we evaluated Hgs individually as stages 1, 2, 3 and some new informations were obtained. While McKenna et al. [2, 24] defined stage 1 Hgs by CD34 and stage 3 by CD20 intensity at a level of mature B-lymphocytes, in this study we defined stage 1 by both CD34 and nTdT expression and extended the definition of stage 3 Hgs for CD10-positivity of brightly stained CD20 cells. Evaluating Hgs individually as stages 1, 2, and 3 in this study we also properly showed flow cytometric analysis of their sIg maturational sequences [Figure 2].

The presented study based a homogeneous group of patients (mainly children), all of them were after finishing complete therapy for AL, they were mostly B-ALL, and mainly children. Complete pattern of all three individual Hg stages was present in all these patients. Maturation spectra of sIgκ and sIgλ light chains and IgM, IgA heavy chains in all three stages of Hgs are presented, as precise reports on sIgs and their incidence on Hgs are scarse and contradictory. Majority of them was obtained by immunohistochemical stains, enabling to compare them with our results detected by multiparameter flow cytometry. The Igs expression paralles CD20 expression. SIg of light and heavy chains were completally absent in stage 1 Hgs and their expression increased through stage 2 to 3; IgM was expressed similarly (Figure 2). Light Ig chains were expressed in a polytypic way. In one BM aspirate (Figure 3) the expression of Hg sIgs was in B-ALL patient present in coexistence with small pathological gate. Coexistence of leukemia cells did not hamper the identification of Hg stages and normal B-lymphocyte maturation with respect to Ig maturation sequence.

Using multiparameter FC analysis more exact information on individual stages of benign B-lymphocytes/hematogones during and after chemotherapy could be obtained. The ability to sensitively differentiate the identities of all Hg stages from leukemia blasts, particularly in B-ALL patients, during and after therapy substancially improves the detection of residual disease.

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