Immunophenotypic Analysis of Bone Marrow B Lymphocyte Precursors (Hématogones) by Flow Cytometry

N BRAHAM JMILI, S NSAIBIA, MC JACOB, H OMRI, MA LAATIRI, S YACOUB, Y BRAHAM, M AOUNI, M KORTAS

ABSTRACT: The aims of this flow cytometry study were to quantify B lymphoid precursors known as hématogones across age and clinical conditions and to study the immunophenotypic profile of these benign immature B cells. A total of 406 consecutive marrow specimens were analyzed for hématogones using 4-color flow cytometry during a 19 month period (60% males and 40% females). The age range was 3 months to 89 years. Hématogones were present in 80% of the specimens. Morphologic analysis of the smears from each patient showed small numbers of hématogones (<13% of total cellularity). The B cell population was defined by CD 19+ CD45 bright positivity, coexpression of other B lineage markers: CD20, CD22, CD10, CD29, CD38 and CD58 in addition to HLA-DR and CD34. In our study we found a significant decline in hématogones with increasing age but a broad range was found at all ages. Marrow from some adults contained relatively high numbers. Diagnosis in these patients included cytopenias, infections, and neoplastic diseases. Distinction of hématogones is critical for disease management particularly after therapy of paediatric B acute lymphoblastic leukaemia to monitor for minimal residual disease.

N Braham Jmili is **professor**, *Faculty of Pharmacy, University of Center, Monastir, Tunisia.*

S Nsaibia is a **physician**, *Faculty of Pharmacy, University of Center, Monastir, Tunisia.*

MC Jacob is a **physician**, *EFS Rhone-Alpes, Department of Cellular Immunology, Grenoble, France.*

H Omri is **professor**, *Department of Clinical Haematology. Hospital Farhat Hached. Sousse. Tunisia.*

MA Laatiri is **professor**, *Department of Clinical Haematology. Hospital Farhat Hached. Sousse. Tunisia.*

S Yacoub EFS is **professor**, *Faculty of Pharmacy, University of Center, Monastir, Tunisia.*

Y Braham is **professor**, *Faculty of Pharmacy, University of Center, Monastir, Tunisia.*

M Aouni is **professor**, *Faculty of Pharmacy, University of Center, Monastir, Tunisia.*

M Kortas is **professor**, *Faculty of Pharmacy, University of Center, Monastir, Tunisia.*

Address for Correspondence *Braham: JMili Néjia, Laboratory of Haematology, HOSPITAL FARHAT HACHED, 4000 Sousse, Tunisia, Tel: 00216 98 68*

52 08, Fax: 00216 73 22 67 02. Email: jmilinejia @yahoo.fr.

INTRODUCTION
Hématogones were first described in the 1930s as lymphoid appearing cells on sternal marrow aspirates[1]. Following the early morphologic descriptions, hématogones were generally regarded as undifferentiated stem cells but their real nature was unknown for more than half a century[2]. In the 1980s new information about their biologic significance began to emerge. Integration of indirect immune-fluorescence and immunoperoxidase staining with morphologic assessment and the terminal deoxy-nucleotidyl transferase (TdT) positive cells found in normal bone marrow were identified as hématogones[3]. Later it was revealed by flow cytometry that hématogones expressed other B cell precursor associated antigens[3,4].

Hématogones may morphologically resemble the neoplastic lymphoblasts of precursor B cells of acute lymphoblastic leukaemia (ALL) and their immune-phenotype also has features in common with neoplastic lymphoblasts[5,6].

Distinction in the bone marrow of benign B-lymphocyte precursors known as hématogones from neoplastic lymphoblasts of ALL is critical for disease management (in post-chemotherapy and post-bone marrow transplant regenerating marrow)[6,7].

The purposes of this prospective multiparametric flow study were:
- To quantify hématogones across age groups and a spectrum of clinical conditions.
- To study the immunophenotypic profile of hématogones: the spectrum of antigen expression typical for normal evolution of B lineage precursors.
- To compare their immunophenotype with that of neoplastic lymphoblasts reported in the literature.

MATERIALS AND METHODS
A prospective 4-color flow cytometry analysis of hématogones was performed during a 19–month period at the Faculty of Pharmacy, University of Center, Monastir. During this period 450 bone marrow specimens were submitted; 44 of these could not be assessed for hématogones for a variety of technical reasons, including lack of adequate numbers of viable cells in the sample.

Patients were separated by gender and age group of younger than 3 years, 3 to 5 years, 6 to 15 years, 16 to 50 years and 50 years and older.

Patients were also separated according to clinical information.

CYTOLOGICAL INVESTIGATION
- Aspiration of bone marrow: sternum punction in adults and iliac punction in children in ethylenediamine tetraacetic acid tube.
- Automatic staining of bone marrow smears by using the HEMATEK slide stainer (AMES company) and a HEMATEK bloc colorant stain pack (Bayer Diagnostica).
- Cytomorphologic examination of the bone marrow slides separately by 2 morphologists into healthy and pathological samples.

FLOW CYTOMETRY METHODS
Cell Isolation: Preparation
- Cell counts of the bone marrow specimens were first done on the Coulter MAXM blood cell counter.
- The cells were incubated for 15 minutes in the dark with each of the conjugated mono-clonal antibodies.
- Erythrocyte lysis: erythrocytes were lysed using lysing solution (optilyse A 11895 - Beckman Coulter) according to the manu-facturer's instruction.
- Following the lysis step, the samples were washed two times with Phosphate buffered saline (PBS).

- Centrifuge for 5 minutes at 200x g, remove the supernatant by aspiration and shake the cell pellet carefully.
- The cell pellet was conserved at + 4 °C.
- Cells were resuspended in PBS for acquisition.

Antibodies :
- Antibodies to the following antigens were used to specifically profile B cell
- precursors (Coulter- Immunotech):
- CD10 Fluorescein Isothiocyanate(FITC), CD34 (FITC), HLA DR (FITC), CD29 (FITC), CD38 R-Phycoerythrin, CD22 (PE), CD20 (PE), CD58 (PE), CD 19 Allophycocyanin (APC), CD45 R-Phycoerythrincyanin 5.1(PC 5).
- Four 4-color combinations were used in each case (Table 1).
- Data analysis was done using a FACS Calibur Flow cytometer (Becton Dickinson) with Cell Quest Pro Software (Becton Dickinson). For each experiment, 500,000 cells were analyzed.

Table 1. Protocol of flow cytometry analysis.

	FITC fluorescein isothiocyanate	PE R-Phyco-erythrin	APC Allophy-cocyanin	PC5 R- Phycoeryth -rincyanin 5.1
1	IgG1	IgG1	CD19	CD45
2	CD10	CD38	CD19	CD45
3	CD34	CD22	CD19	CD45
4	HLA DR	CD20	CD19	CD45
5	CD29	CD58	CD19	CD45
Volume	5 µl	5 µl		5 µl dilution in PBS at (1/5)

Flow cytometry interpretation
Samples were acquired with a three color flow cytometer. Distinct cell populations (clusters) were identified based on any combination of forward and orthogonal light scatter properties and fluorescence intensity with various antibody combinations. Each specimen's event clusters were considered positive or

negative when compared with the degree of the same specimen stained with the isotypic control antibody.

RESULTS
Morphologic features and distribution:
The specimens submitted for flow cytometry were systematically studied for morphology. Hématogones were frequently present in sufficient numbers to be recognized (1 to 13%). There was a spectrum of size and the exhibited features varied from mature lymphocytes to lymphoblasts of ALL.

They varied from 10 to 20µ in diameter, with smaller cells predominating (Figure 1). The nucleus was round, oval with some indentation. The nuclear chromatin was condensed but homogenous. Nucleoli were absent or small and indistinct. There was scant or no discernible cytoplasm, but it was clearly seen in some of the cells. When present, the cytoplasm was moderately to deeply basophilic and devoid of inclusions, granules, or vacuoles. A relatively small percentage cells were indistinguishable morphologically from the lymphoblasts of ALL. Chromatin was fine and contained no obvious clumps. Some nucleoli were large and prominent. The presence of one or more nucleoli indicated immaturity.
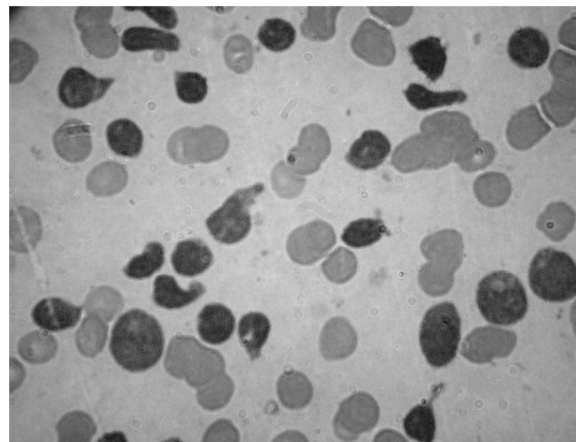
Figure 1. Photograph taken with a 100x objective lens in a bone marrow aspirate smear from a young patient with Evans syndrome and increased (35%) hématogones. Many of the hématogones exhibit cytological features that are common for neoplastic lymphoblasts of acute lymphoblastic leukaemia.

Immunophenotypic features of hématogones:
A total of 406 bone marrow specimens were analyzed for hématogones using 4-color antibody combinations (Table 1).

In 325 (about 80%) of the 406 bone marrow specimens, hématogones were identified by flow cytometry. Selection of B precursors was done by characterization of CD19 + CD45 flow cells (Figure 2 and 3). In all instances the hematogone population exhibited a typical complex spectrum of antigens of B-lineage precursors. In our study the interpretation of flow cytometric data demonstrated that hematogone proliferations exhibited a complex spectrum of antigen expression that defineed the normal antigenic evolution of B cell precursors with predominance of intermediate and mature B lineage cells (Figure 4). The B cell subpopulation was defined by CD 19+ CD45 bright positivity and coexpression of other B lineage markers: CD20, CD22, C10, CD29, CD38 and CD58 in addition to HLADR and CD34.
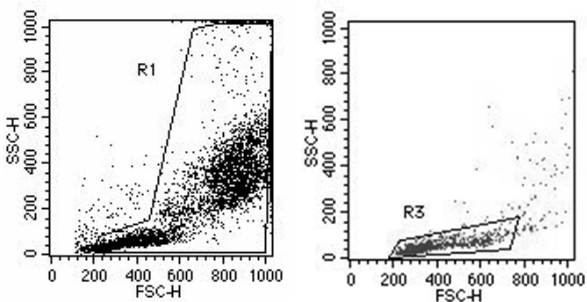
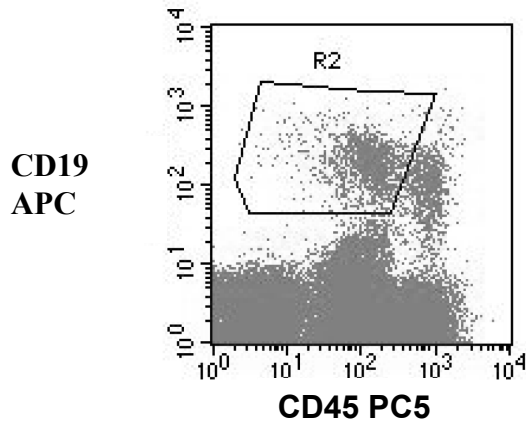

Figure2. Selection of mononuclear cells.

Figure 3. Gating strategy for detection of benign B precursor cells (hématogones) (CD19+ CD45 low)
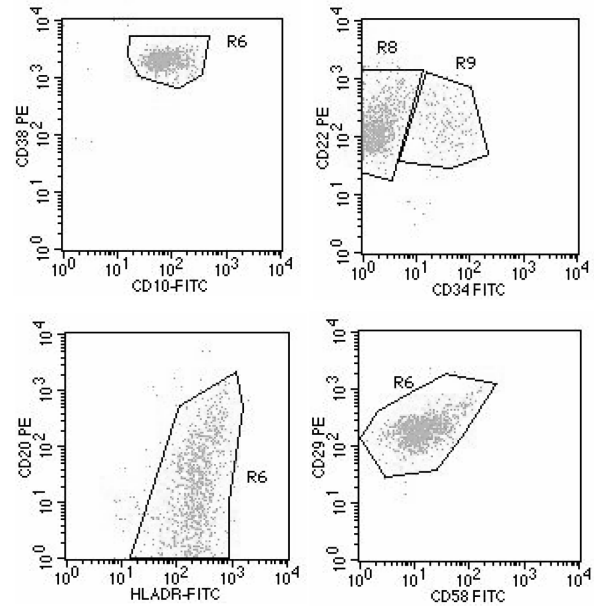

Figure 4. Intensity expression of markers that characterizes benign B cell differentiation(CD38, CD10, CD22, CD34, CD20, HLA DR, CD29, CD58).

Numerical variations of hématogones:
Associations of the percentage of bone marrow hématogones with age and sex were analyzed (Table 2).

Table 2. Bone marrow hématogones by percent age group for the 406 specimens.

Patients (age, years)	< 3	3-5	6-15	16-50	>50
Number / All specimens	8	15	72	168	143
Number /positive specimens	8	13	51	128	125
Hématogones (intervals)	[6-20]	[5-15]	[5-35]	[1-27]	[0,5-5]
Hématogones (mean percent)	10,5	4,3	3,9	2,8	1,2

A total of 406 bone marrow specimens from patients were analyzed. Sixty percent of the specimens were from males and forty percent from females. Ages

ranged from 3 months to 89 years (mean 47 years, median 50 years).

Eight specimens were from patients aged less than 3 years, 15 from patients aged 3 to 5 years, 72 from patients aged 6 to 15 years, 168 from patients aged 16 to 50 years, and 143 from patients older than 50 years.

Clinical conditions with increased hématogones

In our study, the hématogones were abundant (>5% of bone marrow cells) in several clinical conditions (Table 3).

Table 3. Clinical conditions with increased hématogones for the 406 specimens.

Disease	Number of cases
Neoplastic disease:	70/406 cases
Known myeloid leukaemia	50
Other neoplasias (Nonhaematopoietic neoplasms)	20
Cytopenia:	257/406 cases
Idiopathic thrombocytopenia purpura	86
Megaloblastic anaemia	74
Others	97
Infectious disease:	79/406 cases

DISCUSSION

Hématogones were identified by 4-color flow cytometry using optimal antibody combinations in many bone marrow samples [3,6,7]. Bone marrow hématogones were separately assessed as hematogone 1 populations of early stage and hématogones 2 of mid-stage precursor B cells, respectively. In some (about 30%) of the hématogones, a third type of

hématogones could be assessed in the bone marrow samples (Figure 4)[6]. Our study showed that intermediate hématogones predominated. Increased information about benign B lymphocyte precursors, especially the existence of a third type hématogones could provide a basis for better discrimination of B leukaemia cells even in very small amounts. In a

multidisciplinary study, Rimsza, has demonstrated that hematogone-rich lymphoid proliferations exhibit a spectrum of B- lymphoid differentiation of antigen expression with predominance of intermediate and mature B lineage cells[8]. Flow cytometry revealed in this study that intermediately differentiated cells (CD10+,CD19+) predominated and followed in frequency by CD20+ [8].

Hématogones may morphologically resemble the neoplastic lymphoblasts of precursor B ALL, and their immunophenotype also has features in common with neoplastic lymphoblasts. Thus, distinction of hématogones and neoplastic lymphoblasts of B cells present in bone marrow may cause diagnostic problems due to their morphologic and immunophenotypic similarities with neoplastic lymphoblasts of acute lymphoblastic leukaemia [5,6,9,10].

In the medical literature that we reviewed, the neoplastic lymphoblasts in precursor B ALL deviated from the normal B-lineage maturation spectrum and exhibited maturation arrest and over-, under-, and asynchronous expression of antigens observed on normal B-cell precursors. They often aberrantly expressed myeloid-associated antigens [5].

Hematogone populations always exhibit a continuous and complete maturation spectrum of antigen expression typical of the normal evolution of B-lineage precursors and they lack aberrant or asynchronous antigen expression[5]. (Table 4) Hematogones are precursors which were defined by CD19 positivity and CD45 bright. The expression of antigen immaturity includes HLA DR and CD34, and the co-expression of the more mature markers CD19, CD20, CD22. These cells are blended and confused with those of mature B lymphocytes (CD10 negative) on CD45/SSC and could be better recognized on CD10 gating[6].

Leukemic cells can be distinguished from normal haematopoietic cells on the basis of morphology, of chromosomal or molecular abnormalities and immunophenotype. With flow cytometry using opti-

mal antibodies in combination, the distinction can nearly always be made. However, we have to empha-

Table 4. Maturational sequence of bone marrow B cell precursors (hématogones). Stage 1 hématogones correspond to the least mature (top horizontal row). Stage 2 includes middle rows and stage 3 the bottom hematogone row. Mature marrow B lymphocytes are shown for comparison(6).

Hématogones

Stage 1

TdT	CD34	CD10	CD19	CD22	CD38		
				(dim)	(bright)		

Stage 2

		CD10	CD19	CD22	CD38		
				(dim)	(bright)		
		CD10	CD19	CD22	CD38	CD20	Slg*
				(dim)	(bright)	(dim)	

Stage 3

		CD10	CD19	CD22	CD38	CD20	Slg*
				(dim)	(bright)		

Mature B Cells

			CD19	CD22	CD38	CD20	SIg*
						(bright to négative)	

* The appearance of surface immunoglobulin is variable among individual cells occurring from shortly before to after acquisition of high level of CD20 expression.

size the difficulties in distinguishing these cells from residual marrow blasts after chemotherapy.[9,10,11]

Hématogones were identified by 4-color flow cytometry using optimal antibody combinations in most bone marrow specimens. They were more commonly found in higher numbers in children and there was a general decline in hématogones with increasing age[12,13,14]. They are often increased (> 5%) in regenerating marrow and in some clinical conditions [3,4,6].

In our study there was a significant decline in hématogones with increasing age, but a broad range was found at all ages, although, some adult's bone marrow contained relatively high numbers (Table 2 and 3).

In a study by Kallkury, flow cytometric analysis revealed 1% to 20% precursor B cells based on expression of 1 or more pan B cell antigens in addition to CD10, CD34 and terminal deoxy-nucleotidyl transferase (TDT) [11]. In Caldwell's study hématogones were most commonly observed in young children, comprising up to 21% of marrow cells in normal infants [15].

It has been reported that the number of hématogones in bone marrow is variable; the hématogones are present in higher numbers in children and they are often increased in regenerating marrow and in some clinical conditions particularly in patients with cytopenias and neoplastic diseases[6,16,17]. It has also been reported that there is a decline in the mean percentage of hématogones with increasing marrow involvement by neoplastic cells [17]. The reason for the decline is uncertain but may relate to encroachment on the hematogone compartment by the neoplastic infiltrate: alteration of factors that regulate B lymphocytogenesis may also play a role. A study has shown that even though total hématogones may be decreased there is an increased proportion of stage 3 hématogones in marrow involved by lymphoma or leukaemia compared to un-infiltrated marrows [18].

Furthermore, hématogones are the predominant lymphoid population in the bone marrow of preterm infants (for10 to 60%; mean = 34%) of all cells. Flow cytometry revealed a level of 3.8 % of immature cells in a < 1 week- old neonate and 25.7% in a 19 week old infant [9].

They are reported to occur in large numbers in some healthy infants and young children and in a variety of diseases in both children and adults [12,13,14,19].

Hématogones may be particularly prominent in the regeneration phase following chemotherapy or bone marrow transplantation and in patients with autoimmune and congenital cytopenias, neoplasms, and acquired immunodeficiency syndromes. In some instances they constitute 5 % to more than 50 % of cells [6,20,21,22]. Immune mediated thrombocytopenia is a

clinical condition characterized by increased platelet destruction due to the sensitization of platelets by antibodies. A statistically significant increase in the percentage of hématogones was demonstrated in their bone marrow. An increased percentage of hématogones was demonstrated; with a mean of 18+/- 15.2%, CD19+ with a mean of 27+/- 16.3% and CD 34+ with a mean of 18+/- 15.2%. This could be the sequence of an immunological response to the cause which determinates the disease, or the regeneration of the stem cell compartment following transient damage [23,24].

The presence of benign immature B cells has been noted to interfere with the flow cytometric analysis of cases of suspected acute lymphoblastic leukaemia because their immunophenotype (positive for CD19, CD10, CD34 and terminal deoxynucleotidyl trans-ferase) is similar to that of pre B cells lymphoblasts and they simulate acute lymphoblastic leukaemia or lymphoma [20,21,25].

The percentage of marrow hématogones may fluctuate with disease status or persistent elevations may occur. Persistent elevations have been observed for 2 years following cessation of chemotherapy for ALL by one group of investigators and another group found elevations for more than a year following marrow transplantation [26,27,28].
The presence of hématogones in clinical samples should be recognized so as not to adversely influence prognostic studies [22, 25]. Flow cytometry is reported to distinguish between these cell populations in nearly all instances

Identification of normal hématogones B contribute to better clarification of the detection of small numbers of blasts B of acute lymphoblastic leukaemia [29,30,31,32].

In conclusion, these findings suggest that it is important to continue this study by flow cytometric analysis of the lymphoblasts of ALL with the same 4 combinations of antibodies in order to clarify the optimal combination which clearly distinguishes B leukemic cell from hématogones. Such comple-

mentary investigations are necessary for the recognition of early relapsed ALL and disease progression. Thus these differences in hématogones and lymphoblasts of ALL would be very important and could be utilized for analysis of minimal residual disease after chemotherapy treatment of B ALL.

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