

Multiparameter flow cytometry to detect hematogones and to assess B-lymphocyte clonality in bone marrow samples from patients with non-Hodgkin lymphomas

Giovanni Carulli,¹ Virginia Ottaviano,¹ Valentina Guerri,¹ Stefano Giuntini,¹ Paola Sammuri,¹ Eugenio M. Ciancia,² Antonio Azzarà¹

¹Division of Hematology, Department of Clinical and Experimental Medicine, Santa Chiara Hospital, University of Pisa;

²Second Division of Pathology, Pisa University Hospital, Italy

Abstract

Hematogones are precursors of B-lymphocytes detected in small numbers in the bone marrow. Flow cytometry is the most useful tool to identify hematogones and, so far, 4-color methods have been published. In addition, flow cytometry is used in the diagnosis and follow-up of lymphomas. We developed a flow cytometric 7-color method to enumerate hematogones and to assess B-lymphocyte clonality for routine purposes. We evaluated 171 cases of B-cell non-Hodgkin lymphomas, either at diagnosis or in the course of follow-up. By our diagnostic method, which was carried out by the combination K λ /CD20/CD19/CD10/CD45/CD5, we were able to detect hematogones in 97.6% of samples and to distinguish normal B-lymphocytes, neoplastic lymphocytes and hematogones in a single step. The percentage of hematogones showed a significant inverse correlation with the degree of neoplastic infiltration and, when bone marrow samples not involved by disease were taken into consideration, resulted higher in patients during follow-up than in patients evaluated at diagnosis.

Introduction

The ontogenesis of B-lymphocytes in humans takes place in the bone marrow.^{1,2} The first recognizable B-cell precursors are called hematogones (HG) and are lymphoid-appearing cells. Their morphology, in the first stages of development, is similar to that of lymphoblasts, while more mature HGs resemble mature B-lymphocytes. Although HG detection is possible by light microscopy in bone marrow histological samples and/or aspirates,³⁻⁵ the most precise tool to identify and enumerate

them is represented by multiparameter flow cytometry (MFC), which allows to characterize various stages of development of HGs and to study all phases of B-lymphocyte ontogenesis.^{5,6}

HG phenotype is characterized by a dynamic evolution of antigens associated with B-cell development, with progressive down-regulation of CD34, CD10 and CD38, and progressive up-regulation of CD45, CD20, CD22 and surface immunoglobulin light chains. Using MFC, three main stages (stage 1, 2 and 3) of HGs can be detected, and they can be distinguished from the final stages of B-cell ontogenesis, which includes both transitional and mature, naïve B-lymphocytes.

Several papers, dealing with HG recognition and enumeration, have so far been published, and recent interesting reviews have provided an extensive survey of such a topic.^{5,6} To the best of our knowledge, all previous investigations were carried out by 4-color MFC, requiring the organization of more than one antibody combination, without the possibility of detecting HGs and assessing clonality of mature B-lymphocytes in the same sample.

In the current paper we describe a rapid, simple 7-color tube which can be used for routine purposes and allows to identify normal and neoplastic B-lymphocytes, to assess their clonality, and to detect and enumerate HGs. Since our laboratory is particularly involved in hematological malignancies diagnosis, we applied this diagnostic MFC tube to samples obtained from patients suffering from B-cell non-Hodgkin lymphomas (B-NHLs). The current paper deals with 171 consecutive cases and includes patients in different phases of disease (at diagnosis, under chronic observation), either with or without bone marrow involvement by disease.

Materials and Methods

Patients

One hundred and seventy-one patients, suffering from various types of B-NHL were studied: 95 were males and 76 females, with age ranging 20-83 (Table 1). B-NHL subtypes were diagnosed according to the 2008 WHO classification.⁷ Ninety-five patients (mean age 60.58, SD 13.39, SEM 1.37, range 20-83) were studied at diagnosis, when bone marrow samples were obtained in the course of disease staging. Seventy-six patients (mean age 60.63 ys, SD 11.24, SEM 1.16, range 38-82) were evaluated during follow-up, after at least 12 months from terminating chemotherapy. Both bone marrow biopsy (BMB) and myeloaspirate (MA) were obtained from all patients, in order to carry out synchronous histological and cytological evaluation and to analyze aspirate samples by MFC.

Correspondence: Giovanni Carulli, Division of Hematology, Santa Chiara Hospital, via Roma 67, 56126 Pisa, Italy.

Tel. + 39.050.992731. Fax: +39.050.993378.

E-mail: giovannicarulli@alice.it

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Myeloaspirate evaluation

MA smears, stained by May-Grünwald-Giemsa, were observed at light microscopy in order to identify hemodiluted samples, which were not considered for our study.

Bone marrow histology

Bone marrow trephines were fixed in Myelodec® reagent A (Bio-Optica, Milan, Italy) for 2 hr, decalcified, embedded in paraffin, and cut into 3-5 μ m sections. Morphological evaluations were carried out on hematoxylin-eosin, Giemsa, and Gordon-Sweet for reticulin-stained sections. Immunohistochemical stainings were performed using a peroxidase-based system and included antibodies specific for CD20/L6, CD3/PS1, CD5/4C7, CD23/1B12, CD45, CD79a, bcl-2/100-D5, cyclin-D1 (DSC-6), CD43, CD10, CD1a/EP3622, CD4/SP35, CD8/SP57, CD30/Ber-H2, CD45RO/UCHL-1, CD56/123C3, CD57/NK-1, CD68/KP1, CD68R/PGM-1, Granzyme B polyclonal, MUM-1/MRQ-43, lysozyme/polyclonal, gliophorin A/GA-R2, myeloperoxidase/polyclonal, and polyclonal antibodies directed to immunoglobulin γ and μ heavy chains, and κ and λ light chains. A BenchMark automated Slide Stainer (Ventana, Milan, Italy) was used.

Flow cytometry reagents and devices

MFC was carried out by a FacsCanto II cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) equipped with three lasers (488, 633, 405 nm) and capable of analyzing combina-

tions of the following fluorochromes: FITC, PE, PerCP-Cy5.5, PE-Cy.7, APC, APC-Cy.7, Am-Cyan/Horizon V500, Pacific Blue/Horizon V450. Data were analyzed by the FACS Diva software (Becton Dickinson). The following monoclonal antibodies (MoAbs), all purchased from Becton Dickinson, were used: CD45, CD3, CD4, CD5, CD8, CD10, CD11c, CD16-56, CD5, CD19, CD20, CD22, CD23, CD25, CD34, CD38, CD43, CD79b, CD81, CD103, CD200. For clonality assessment, F(ab')₂ polyclonal rabbit antibodies directed to human immunoglobulin K and λ light chains, purchased from Dako, were used. Different combinations of antibodies were used, in order to identify HGs, normal and neoplastic B-lymphocytes, minimal residual disease. The setup of the cytometer was carried out by the BD cytometer setup and tracking beads kit (Becton Dickinson) and fluorescence compensation was performed automatically.

Flow cytometric detection of hematogones and simultaneous clonality assessment

Myeloaspirate samples (50 μ L), were subjected to preliminary erythrocyte lysis by NH₄Cl (5 min), followed by two washings with PBS (300 g for 5 min). The cell pellets were pre-incubated with heat-inactivated fetal calf serum (50 μ L, 15 minutes). B-lymphocyte clonality assessment and HG detection and enumeration were carried out by a single, seven-color tube. In 50 cases the antibody panel included: K/FITC; λ /PE; CD20/PerCP-Cy5.5; CD5/PE-Cy.7; CD10/APC; CD19/APC-Cy.7; CD45/Am-Cyan. In the remaining samples, the antibody panel included: K/FITC; λ /PE; CD20/PerCP-Cy5.5; CD19/PE-Cy.7; CD10/APC; CD45/APC-Cy.7; CD5/Horizon V450. Saturating amounts of antibodies (FITC- and PE-conjugated antibodies, 7 μ L; for the other

ones, 5 μ L) were used. After incubation for 20 minutes, samples were processed by acquisition of 100.000 events and a well-defined cluster of at least 10 cells in the CD45/SSC display was considered as suitable for analysis, thus allowing a 0.01% sensitivity. In preliminary tests, additional tubes were organized, in order to obtain a more detailed analysis of HGs, using the following MoAb combinations: CD45/CD10/CD19/CD20/CD34; CD45/CD19/CD22/CD34/CD38.

Routine flow cytometric analysis of B-cell non-Hodgkin lymphomas

Routine MFC assays were used for diagnostic purposes by means the following MoAb combinations: i) CD38,CD4,CD8,CD5,CD19,CD16-56,CD3,CD45 (orientation tube, identification of CD19+/CD5+ lymphocytes; ii) CD19,CD20,CD22,CD79b,CD5,CD45,CD23 (for the differential diagnosis of B-NHLs;) iii) CD19,CD20,CD5,CD200,CD25,CD11c,CD103,CD45 (for the differential diagnosis between hairy cell leukemia and marginal zone lymphomas, and among CD5+ B-NHLs; iv) CD45,CD19,CD20,CD5,CD43,CD81 (for the diagnosis of CLL and identification of minimal residual disease). Analysis was carried out by acquiring 100.000 events/tube and, considering as suitable a well-defined cluster of 10 cells in the CD45/SSC display, sensitivity was 10⁻⁴. Criteria for bone marrow involvement were: clonal K or λ restriction (reference values: 0.5:1-3:1); specific immunophenotype (such as the CD19+/CD20+/CD103+/CD11c+/CD25+/CD200+ co-expression in hairy cell leukemia); typical immunophenotypic pattern (such as CD19+/CD5+/CD23+CD200+/CD43+/CD20+^{weak}/CD81-/CD79b- in CLL; CD19+/CD5+/CD23-/CD20+^{bright}/CD79b+/CD200- in MCL, or CD10+/CD20+CD19+^{weak}/CD43- in follicular lymphoma).⁸⁻¹⁰

Statistics

Means, standard deviations and standard errors of means were calculated by routine methods. When necessary, the Spearman correlation test was used. Differences were considered as statistically significant with P<0.05.

Results

Simultaneous identification of hematogones and mature B-lymphocytes

Figure 1 is representative of the single-tube seven-color analysis of a normal bone marrow sample (in this case, obtained from a patient with a follicular lymphoma limited to a single lesion of the skin). A sequential gating analysis was carried out. The first gate (P1, Figure 1) was set to include both lymphocytes and events within the so-called *blast gate*,^{11,12} and the second gate (P2, Figure 1) included CD19+ cells. In several samples, as shown in this case, a distinct CD19+/CD5+^{weak} B-cell population could be gated (P3). In the following cytogram (Figure 1), the P2 population was found to be composed of three different populations: HGs, with either negative or heterogeneously positive CD20 (P4 and P5, respectively) and mature B-lymphocytes (P6), which were polyclonal, while the two HG subsets lacked surface immunoglobulin light chains. The cell population with CD19+/CD5+^{weak} co-expression (P3) was found to lack CD10 and to be polyclonal and CD20+, thus resulting similar to mature B-lymphocytes. Finally, back gating of the four B-cell subsets demonstrated a different CD45 expression (Figure 1), with increasing positivity starting from the P4 population, which resulted the most immature. CD5+^{weak} mature B-lymphocytes were found within the lymphocytic gate.

Preliminary tests to dissect the hematogone population

Figure 1 shows the different expression of additional markers shown by HGs and mature B-lymphocytes, after CD19 gating (P2, Figure 1). The most immature HG subset, called stage 1 HGs, showed co-expression of CD19,CD34, CD10^{bright},CD38^{bright}. HGs negative for CD34 resulted positive for CD10 and CD38. Mature B-lymphocytes were negative for CD34 and CD10, and resulted to be negative or weakly positive for CD38, with heterogeneous expression.

Such additional tests showed that the CD10+/CD20- HG subset consisted of type 1 and type 2 HGs (as demonstrated in Figure 1) and that all CD34-negative HGs were similar in terms of CD38 and CD22 expression.

We found that the prevalent HG subset was CD34-/CD20- (stage 2 HGs) and accounted for about 60% (mean values), while stage 1

Table 1. General characteristics of patients.

Disease	Males (n=95)	Females (n=76)	Total (n=171)	Age, years (range)
FL	24	34	58	20-82
DLBCL	21	14	35	23-83
MZL	14	12	26	45-80
CLL	17	6	23	46-80
HCL	6	0	6	56-74
MALT	1	4	5	45-74
MCL	8	4	12	53-76
LPL	1	1	2	80-81
SLL	2	0	2	45-71
WM	1	1	2	53-73

FL, follicular lymphoma; DLBCL, diffuse large B-cell lymphoma; MZL, marginal zone B-cell lymphoma (including both splenic MZL and nodal MZL); CLL, chronic lymphocytic leukemia; HCL, hairy cell leukemia; MALT, Extranodal marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue; MCL, mantle cell lymphoma; LPL, lymphoplasmacytic lymphoma; SLL, small lymphocytic lymphoma; WM, Waldenström macroglobulinemia.

and stage 3 HGs accounted for about 15% and 25% of all HGs, respectively (data not shown).

Enumeration of hematogones and mature B-lymphocytes and data reporting

According to the above results, using the seven-color tube developed for routine purposes, HGs were enumerated as a whole population which consisted of all CD19+/CD10+ events, irrespectively from CD20 levels, with intermediate CD45 levels and without surface immunoglobulin light chains. Mature B-lymphocytes were enumerated as the cell population consisting of polyclonal CD19+/CD20+/CD5-/CD10- events plus polyclonal CD19+/CD20+/CD5+ events (if present). Data were expressed in term of percentage of the total events acquired. B-cell clonality was expressed as K/λ ratio.

Hematogones and mature B-lymphocytes in non-infiltrated bone marrow samples from B-cell non-Hodgkin lymphoma patients at diagnosis and in patients in follow-up

One-hundred and twenty-nine bone marrow samples (75 at diagnosis, 54 in the course of

follow-up) were found to be not interested by neoplastic infiltration by combination of morphology and immunophenotypic assays. Results are shown in Table 2: we observed that both the percentage of HGs and the percentage of polyclonal B-lymphocytes were higher in patients during follow-up, with a statistically significant difference. In such samples, HGs were always detected and a wide range of values was registered.

Percentages of hematogones and B-lymphocytes in patients with infiltrated bone marrow

Samples with bone marrow involvement by disease were 42 (CLL: 16; MZL: 10; FL: 5; HCL: 3; MCL: 2; SLL: 2; LPL: 2; WM: 2). Neoplastic infiltration was associated with a significant decrease in the percentage of HGs. This reduction was observed essentially in samples with >25% neoplastic infiltration (Table 2) and corre-

Table 2. Percentages of hematogones and B-lymphocytes.

Patients	No.	Hematogones % (range)	B-lymphocytes % (range)	K/λ ratio (range)
At diagnosis, normal bone marrow	75	0.88±0.89* (0.1-5.4)	1.77±1.20** (0.3-6.9)	1-2
Follow-up, normal bone marrow	54 (0.1-4.7)	1.31±1.21* (0.1-12)	3.02±2.63**	1-2.5
Infiltrated bone marrow, all cases	42	0.72±0.80 (0-3.6)	19.26±21.02 (0.12-85.6)	NA
Infiltrated bone marrow <25%	28	0.98±0.86 (0.1-3.6)	7.66±5.75 (0.12-19.2)	NA
Infiltrated bone marrow >25%	14	0.23±0.25*** (0-0.9)	42.48±21.30 (25-85.6)	NA

Data are expressed as percentages±standard deviation. NA, not applicable. *P=0.018; **P=0.002; ***P=0.001 (vs all the other groups).

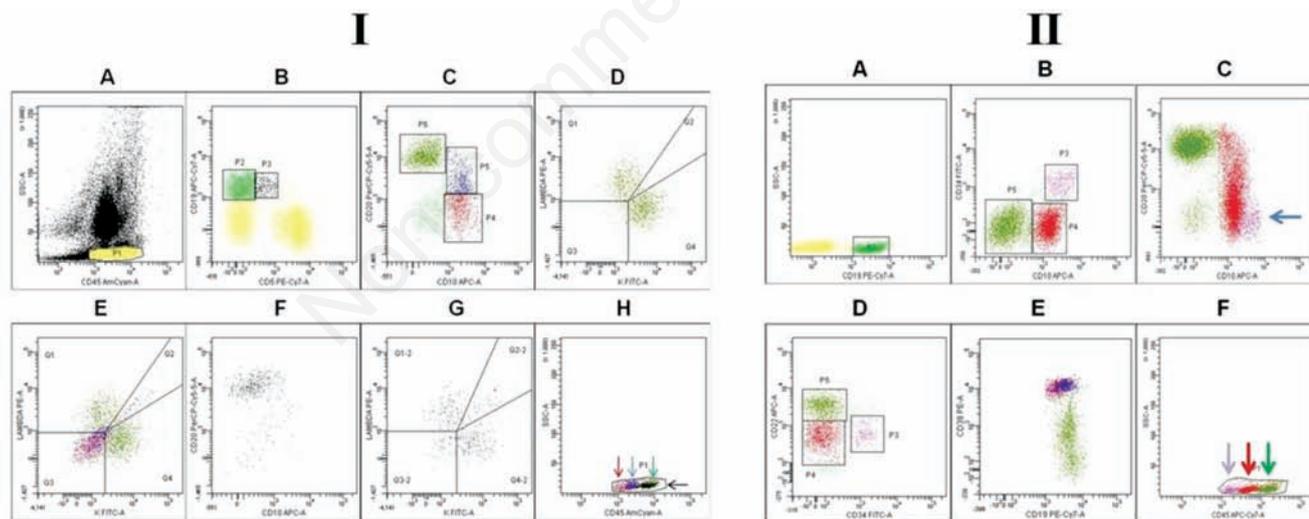


Figure 1. I: simultaneous K/λ analysis and detection of hematogones in a bone marrow sample without lymphomatous infiltration (patient with follicular lymphoma involving a single skin lesion). A) initial gate setting (P1). B) the two gates include CD5-negative B-lymphocytes (P2) and B-lymphocytes with dim expression of CD5 (P3). C) Distribution of CD5-negative B-lymphocytes in the CD10/CD20 cytogram. Hematogones (P4 plus P5) can be observed, along with mature, CD10-negative B-lymphocytes (P6). Hematogones with dim/heterogeneous CD10 expression are 30% of the whole hematogone population. D) K/λ analysis of mature B-lymphocytes, which are polyclonal (ratio: 1.6:1). E) both P4 and P5 cell populations lack surface light chains, in agreement with their relative immaturity. F) the population of CD5-positive B-lymphocytes lacks CD10, and G) is polyclonal (K/λ ratio: 1.35:1). H) CD45/SSC back-gating: an increasing CD45 expression characterizes hematogones and mature B-lymphocytes. The CD5-positive population is localized within the gate of mature lymphocytes. The four arrows show the localization of the respective cell populations. The whole analysis shows: 2.3% mature B-lymphocytes (P3+P6, i.e. 0.8+1.5%); 1.1% hematogones (P4+P5, i.e. 0.77+0.33%). II: additional tube to complete immunophenotyping of the case described in Image I. A) B-lymphocyte gating. B) detection of type 1 HG (P3). P4 includes type 2 and type 3 HG. P5: mature B-lymphocytes. C) CD34+ hematogones show the brightest CD10 expression (arrow). D,E) different pattern of expression of CD22 (D) and CD38 (E) in HG and mature B-lymphocytes. F) back-gating of HG and mature B-lymphocytes in the CD45/SSC cytogram, as shown by the arrows.

lation tests showed a clear relationship between decreasing HG percentage and increasing lymphomatous infiltration (Figure 2).

Failure to detect hematogones in infiltrated samples concerned only 4 patients included in the subset of samples with $\geq 25\%$ bone marrow infiltration. Therefore, the percentage of samples without detectable HGs, using our diagnostic conditions, resulted very low (2.3%). HGs were easily distinguished from both normal and neoplastic B-lymphocytes, as shown in representative cases of CLL and follicular lymphoma (Figure 3).

Samples with hematogone hyperplasia

The percentage of samples with HG hyperplasia, defined as HGs $>3.5\%$, resulted to be very low, since only one sample (patient at diagnosis, uninvolved bone marrow) showed 5.4% HGs, and other 5 samples showed HGs ranging 3.6-4.7. Thus, HG hyperplasia accounted for 3.5% of all samples. Despite the use of a diagnostic tube which included CD5, we were not able to find HGs positive for this molecule, since the B-lymphocyte subset co-expressing this molecule was always found to overlap the phenotype of polyclonal, mature B-lymphocytes and to show polyclonal expression of surface immunoglobulin light chains.

Discussion

Human B-lymphocyte ontogenesis occurs in the bone marrow, starting from a common myeloid-lymphoid precursor, through several development stages. The most immature progenitors of B-lymphocytes are very rare cells and cannot be detected by routine methods.^{1,13}

The first B-lymphocyte precursors recognizable by MFC are called HGs and can be identified by means of combination of some antibodies directed to markers which are down- or up-regulated in the course of evolution towards mature cells.⁶ Using the flow cytometric approach, HGs can be distinguished in three main subsets.¹⁴⁻¹⁸

Stage 1 HGs are characterized by positivity

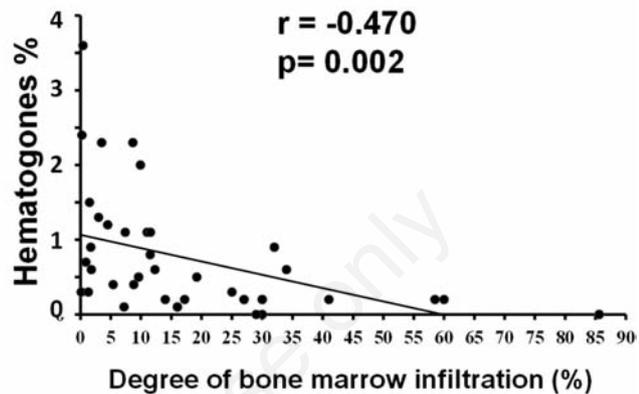


Figure 2. Correlation between hematogones percentage and degree of bone marrow infiltration by neoplastic lymphocytes.

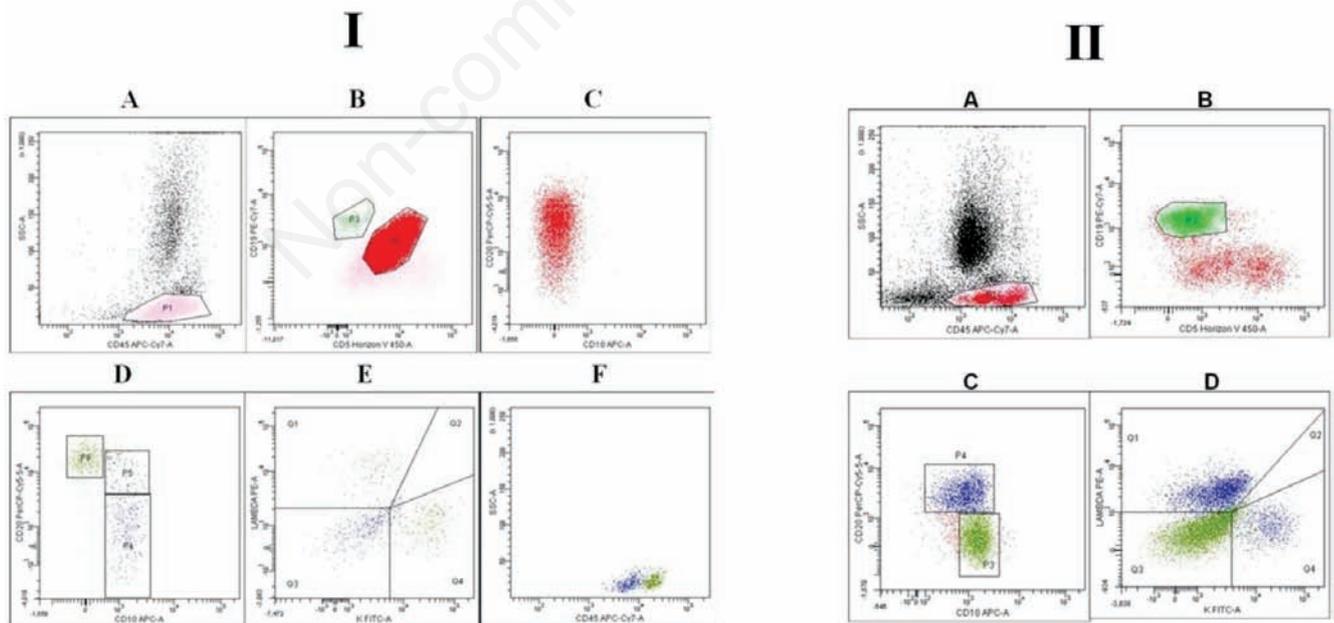


Figure 3. I. A case of CLL. A) initial gate (P1). B) detection of pathologic lymphocytes (P2) and CD19+/CD5- B-cells. C) the neoplastic lymphocytes are CD10-negative. D) CD19+/CD5- are composed of hematogones (P4+P5) and normal B-lymphocytes. E) different K/λ expression by hematogones and normal B-lymphocytes. F) back-gating. The P2 population represents 55% of total events, while P3 accounts for 0.5% of total events (hematogones: 0.2%; normal B-lymphocytes: 0.3%). II: a case of follicular lymphoma. A) initial gate (P1). B) CD19+ cells (10%). C) hematogones (P3, 5%) are present along with a pathologic, CD10+/CD20+ mature B-lymphocyte population (P4, 5%), which shows D) clonal λ excess (K/λ ratio: 0.25:1).

for TdT, CD34, CD19, CD38 and CD10. Stage 2 HGs lack TdT and CD34 and are positive for CD19, CD10, CD38, cytoplasmic IgM. Stage 3 HGs are similar to the previous stage, but show a progressive expression of CD20 and surface immunoglobulin light chains. In this stage initial expression of CD22 occurs, along with a progressive reduction in CD10 expression. The expression of CD45 progressively increases during maturation from stage 1 to stage 3 HGs. According to the current literature, stage 2 HGs are the main population, accounting for about 65% of all the HG population.

The final B-lymphocyte development leads to the formation of mature cells which show the brightest expression of CD45 and are positive for CD19, CD20, surface IgM and IgD. Such cells are polyclonal, show down-regulation of CD38 and lack CD27 (*i.e.*, they are naïve cells). Several bone marrow specimens show the presence of a subset of mature, polyclonal B-lymphocytes which co-express CD5,^{19,20} and have been termed transitional B-lymphocytes.

The bone marrow, however, is a frequent site of localization of B-NHLs. This behavior, which is characteristic of CLL and HCL, concerns variable percentages of the other NHL histotypes.²¹ Bone marrow investigation is one of the steps of disease staging, since evidence for bone marrow involvement results in a diagnosis of stage IV disease and is associated with shortened survival.

MFC is one of the methods currently used to analyze bone marrow samples in B-NHLs and provides important data in order to measure lymphomatous infiltration, to improve differential diagnosis and to detect minimal residual disease.^{8,22,23}

Our laboratory is involved in diagnosis and monitoring of NHLs and routine analysis is carried out by combinations of 7 or 8 antibodies.⁹ Among the various diagnostic tubes, we developed a 7-color method to perform a simultaneous detection of normal B-lymphocytes, neoplastic cells and hematogones, using CD45, CD19, the main B-cell markers which are differently regulated during B-lymphocyte development (CD10 and CD20), antibodies specific for K and λ light chains, and CD5.

Using our technical approach, we were able to detect HGs in 97.6% of cases, with failure only in a few samples with marked neoplastic infiltration. We found that uninvolved bone marrow samples from patients in follow-up, studied at least 12 months from last chemotherapy, showed higher HG percentages than patients with normal bone marrow, but evaluated at diagnosis. The mean percentage of HGs enumerated in patients in follow-up was 1.3% and resulted very similar to the results (1.1%) reported by McKenna *et al.* in 207 specimens evaluated by the same NH_4Cl lysis procedure as ours.¹⁵ Similarly, results were characterized by high standard deviation

values, due to a very wide range. To date, the percentage of HGs in normal subject remains unknown, as discussed by Chantepie *et al.*,⁶ but it is probable that values of 1.1-1.3% might be considered as near to normal figures.

It should be underlined the fact that our patients' population consisted of only NHLs cases and that mean age was about 60 years, with no patients under 20 years. Thus, some differences might be due to patients' characteristics, since children usually show higher HG percentages than adults, and since HGs seem to decline with age.¹⁵

In pathologic, infiltrated bone marrow samples our diagnostic tube was found to distinguish neoplastic lymphocytes from both residual, normal B-lymphocytes and HGs. Thus, with a single analysis it was possible to study B-cell ontogenesis in the course of NHL observation. This approach might be interesting when a prospective analysis of bone marrow samples is required. This could be the case of patients treated with chemo-immunotherapy, when anti-CD20 antibodies (Rituximab) are used,²⁴ and when immunotherapy is chosen as maintenance therapy, for example in follicular lymphoma.²⁵

In such cases, monitoring CD20+ lymphocytes may be useful to optimize maintenance therapy and the use of the single 7-color tube allows to confirm the absence of lymphomatous infiltration.

We were able to distinguish normal from pathologic CD10+ B-cells, as found in cases of follicular lymphoma. In addition, the inclusion of CD5 in the MoAb panel allows to distinguish normal, transitional B-lymphocytes from lymphomatous cells, and this appears to be very useful in all CD5-expressing NHLs. Such a purpose is particularly important when minimal residual disease evaluation is required.²⁶

We were not able to detect CD5-expressing HGs. The existence of this HG subset has been reported previously in cases with HG hyperplasia (defined as HGs more than 3.5%).²⁷

However, we found that in normal, non-infiltrated bone marrow samples, CD5+ B-cells were always polyclonal, CD20+ and CD10-, thus very close to naïve B-lymphocytes from a phenotypical point of view. It should be underlined, however, that CD5+ normal bone marrow B-lymphocytes are usually characterized by dim expression of such a molecule and that fluorochromes selection has to be considered as an important variable that might influence the recognition of CD5+ B-cells.²⁶

Conclusions

In conclusion, MFC performed with the association of seven antibodies appears to be

an useful diagnostic tool in cases of NHLs, when routine evaluation of B-cell ontogenesis and disease assessment are required. The MoAb panel can be expanded up to 8 fluorescences or more, depending on the technical characteristics of cytometers and on MoAb availability. Thus, similar approaches might be proposed to study HGS and B-cell development in other clinical conditions. In fact, there is an increasing interest in studying HGs in different diseases, such as acute myeloid leukemia both at diagnosis and after therapy,^{28,29} in myelodysplastic syndromes,³⁰ in patients undergoing hematopoietic stem cells transplantation.^{4,31}

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