original paper

haematologica 2001; 86:934-940
http://www.haematologica.it/2001_09/0934.htm

Flow cytometry in the bone marrow evaluation of follicular and diffuse large B-cell lymphomas

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Background and Objectives. Bone marrow biopsies are routinely performed in the staging of patients with lymphoma. Despite the lack of evidence for its usefulness, many institutions include flow cytometry (FC) of bone marrow aspirates in an attempt to increase sensitivity and specificity. The aim of this study is to evaluate the usefulness of FC for the assessment of bone marrow involvement by lymphoma in follicular (FL) and diffuse large B-cell lymphomas (DLBCL).

Design and Methods. Seventy-nine bone marrow biopsies from 65 patients diagnosed with FL or DLBCL were examined to compare histology and FC for the assessment of bone marrow involvement by lymphoma.

Results. Bone marrow histology showed involvement (BM^+) in 16 cases (20.3%), lack of infiltration (BM^-) in 52 cases (65.8%) and undetermined or undiagnosed for involvement (BMu) in 11 cases (13.9%). FC was positive for involvement in 28 cases (35.4%) and negative in 51 cases (64.6%). Sixty-five cases (95%) showed concordance between the results of morphology and FC (BM^+/FC^+ or BM^-/FC^-). No BM^+/FC^- cases were observed. Three cases showed discrepant results (BM^-/FC^+). In these 3 cases the molecular studies (PCR) demonstrated clonal rearrangement of the heavy immunoglobulin chain (IgH) and/or bcl2-IgH in agreement with the flow results. Among the 11 cases with BMu, all but 2 were FC^+ and concordance with the PCR results was seen in 9 cases (81.9%).

Interpretation and Conclusions. We conclude that FC is just as sensitive or perhaps slightly more sensitive than histology in the detection of bone marrow involvement in FL and DLBCL. FC studies may be warranted in those cases in which the morphology is not diagnosed. The clinical relevance of the small clonal B-cell population in patients without histologic bone marrow involvement (BM^-/FC^+ cases) remains an open question.

Key words: flow cytometry immunophenotyping, follicular lymphoma, diffuse large B-cell lymphoma, bone marrow.

Routine staging in non-Hodgkin's lymphomas includes evaluation of bone marrow involvement and can affect both prognosis and treatment strategies.[1-7] Traditionally, bone marrow assessment is performed by morphologic examination of the bone marrow biopsies obtained from the iliac bone. There is a consensus that the minimal total length of the bone marrow biopsy should be 2 cm either in unilateral or bilateral iliac bone biopsies.[8] A lower total length of the biopsy samples can affect the sensitivity and lead to underdiagnosis of advanced stages. In spite of these recommendations, it is not always possible to obtain a valuable sample due to the clinical conditions of the patient. In these cases, other techniques such as FC or molecular studies can improve the sensitivity of the bone marrow biopsy. FC immunophenotyping is an important tool in the diagnosis of acute and chronic leukemia. Moreover, it can improve accuracy in the diagnosis of some non-Hodgkin's lymphomas.[9] In spite of its usefulness in diagnosis, not much information is available on its utility in the evaluation of bone marrow involvement by lymphoma.[10-14] Some reports suggest that FC has a good correlation with morphology and that in a few cases there are discrepant results which seem to suggest that FC has marginal value in the routine staging of lymphoma.[9,10] It seems logical to assume that in the cases in which morphologic assessment of bone marrow biopsies shows unequivocal involvement

by lymphoma, then further assessment by FC can be considered to be redundant. Despite the lack of evidence for its utility, FC of bone marrow aspirates are routinely performed in some institutions to improve sensitivity and specificity of the morphologic examination.

The aim of this study is to compare both the FC and histology methods, and to evaluate the usefulness of FC in the assessment of bone marrow involvement by the two most frequent non-Hodgkin's lymphomas diagnosed at our institution, FL and DLBCL.

Design and Methods

Between January 1997 and December 1999, 79 bone marrow aspirates and trephine biopsies were obtained from 65 patients who had been diagnosed as having FL or DLBCL and were morphologically analyzed in parallel and by FC immunophenotyping to detect bone marrow involvement. Pathologic diagnoses were previously assessed in all patients according to the REAL criteria.[15]

Bone marrow specimens

Unilateral trephine biopsy was obtained in all cases from the posterior iliac crest bone. Prior to the biopsy, between 1 to 1.5 mL of bone marrow aspirate was collected in EDTA anticoagulant. It was well mixed and sent for FC analysis. The samples were maintained at controlled room temperature (no more than 25°C) until they were processed (no longer than 24 hours). The bone marrow core biopsy specimens were fixed in B5 fixative and sent for morphologic evaluation. The biopsy length was registered and reported.

Morphologic evaluation

Morphologic evaluation was performed without prior knowledge of FC results. Trephine biopsies were stained with hematoxylin and eosin after standard decalcification and paraffin embedding procedures. Immunohistochemical studies were performed in all bone marrow biopsies. Primary antibodies employed for immunohistochemical procedures were: anti-CD3 (UCHT1), anti-CD45Ro (OPD4), anti-CD20 (L26), anti-BCL2 (124), CD10 (SS2/36), anti-κ light chains (A8B5) and anti-λ light chains (N10/2) supplied by Dako. To visualize the antibody binding we used an alkaline-phosphatase based system (EnVision-Dako®).

The morphologic bone marrow diagnosis was graded as positive or had lymphoma involvement (BM^+), negative or without evidence of involvement (BM^-) and undetermined or undiagnosed for bone marrow involvement (BMu).

Flow cytometry evaluation

The processing and acquisition of the samples was performed according to the US-Canadian Consensus Recommendations on the Immunophenotypic Analysis of Hematologic Neoplasia by Flow Cytometry.[16-18]

For the immunophenotypic analysis of BM cells a standard whole-marrow assay with erythrocyte cell lysis was used for preparing all the bone marrow aspirates. The cells were stained by direct immunofluorescence using monoclonal antibodies conjugated with the following fluorochromes: fluorescein isothiocyanate (FITC), phycoerythrin (PE) and the tandem phycoerythrin-cyanin 5.1 (PC5) (Table 1). A quantity of 1×10^6 cells were stained for each triple antibody combination in a panel, as follows (FITC, PE, PC5): CD8/CD4/CD3, CD19/CD20/CD45, CD3/CD56+16/CD45, CD45/CD34/CD19, κ/λ/CD19. Briefly, 100mL of the bone marrow sample diluted in PBS buffer were incubated for 10 minutes at room temperature in the dark with saturating amounts of fluorochrome-conjugated monoclonal antibodies. After this incubation period, 2 mL of a commercial erythrocyte lysis reagent solution (Ortho Lysis Reagent®) were added to each tube following the specific instructions of the manufacturer, and the samples were incubated again for a further 10 minutes at room temperature in the dark. Afterwards, cells were centrifuged (5 min × 500g) and the cell pellet washed with 2 mL of PBS buffer.

Acquisition was performed in an EPICS-XL MCL (Coulter®) flow cytometer, provided with an argon laser tuned at 488 nm and 15 mW. Acquisition and analysis software was System II (version 2.1) (Coul-

Table 1. Antibodies used in the study for 3 color flow cytometry.

Antigen	*Antibody (clone)*	*Source*	*Label*
CD19	J4.119/ HD37	Immunotech/ Dako	FITC/ PC5
CD20	B9E9	Immunotech	PE
CD3	UCHT	Immunotech	PC5
CD4	13B8.2	Immunotech	FITC
CD8	B9.11	Immunotech	PE
CD45	J.33	Immunotech	FITC/ PC5
CD34	581	Immunotech	PE
CD56	MY31	Becton-Dickinson	PE
CD16	B73.1	Becton-Dickinson	PE
κ/ λ	Goat F(ab')2	Dako	ITC/ PE

FITC: fluorescein isothiocyanate. PE: phycoerythrin. PC5: phycoerythrin-cyanin 5.1.

ter®). Calibration of the instrument was performed daily with labeled fluorospheres (flow-check, Immunotech®). Five parameters were studied: forward scatter (FSC) and side scatter (SSC) on a linear scale together with three fluorescences on the logarithmic scale (FL1, FL2 and FL4). A minimum of 1×10^4 lymphocytes in an SSC/ FSC gate were acquired for determination of B and T cell subsets and a minimum of 5×10^3 B-lymphocytes ($CD19^+$) for κ and λ sIg analysis. In all cases, isotype-matched immunoglobulin with no reactivity against bone marrow cells and the $CD4^-$FITC/$CD8^-$PE/$CD3^-$PC5 combination were used as negative and positive controls, respectively. Both FSC/SSC and CD19/SSC were used in each case as the primary gating methodologies. Further gating strategies were performed as necessary on either lymphoid subpopulations based on cell size and antigen expression. Acquisition data were stored in list-mode files for further analysis.

Criteria for flow cytometry involvement for lymphoma (FC^+) were defined as follows: 1) when the gated B-cells showed sλγ light chain restriction in part of or all of the cell population; 2) when the κ-to-λ light chain ratio was > 3:1 or < 1:1 in the B gated population; 3) when there was a lack of expression of membrane immunoglobulin in the mature B cells defined as $CD45^{bright+}$, $CD20^{bright+}$, $CD34^-$ (Figure 1).

Table 2. Summary of FC results and histologic studies.

	BM+ n (%)	*BM- n (%)*	*BMu n (%)*	*Total n (%)*
FC+	16 (20.2%)	3 (3.8%)	9 (11.4%)	28 (35.5%)
FC-	0 (0.0%)	49 (62.0%)	2 (2.5%)	51 (64.5%)
Total	16 (20.2%)	52 (65.8%)	11 (13.9%)	78 (100%)

BM+: bone marrow morphologically involved by lymphoma; BM-: Bone marrow without morphological evidence of involvement by lymphoma; BMu: bone marrow with histology undetermined or not diagnostic for bone marrow involvement; FC+: bone marrow involved by lymphoma by flow cytometry; FC-: bone marrow not involved by lymphoma by flow cytometry.

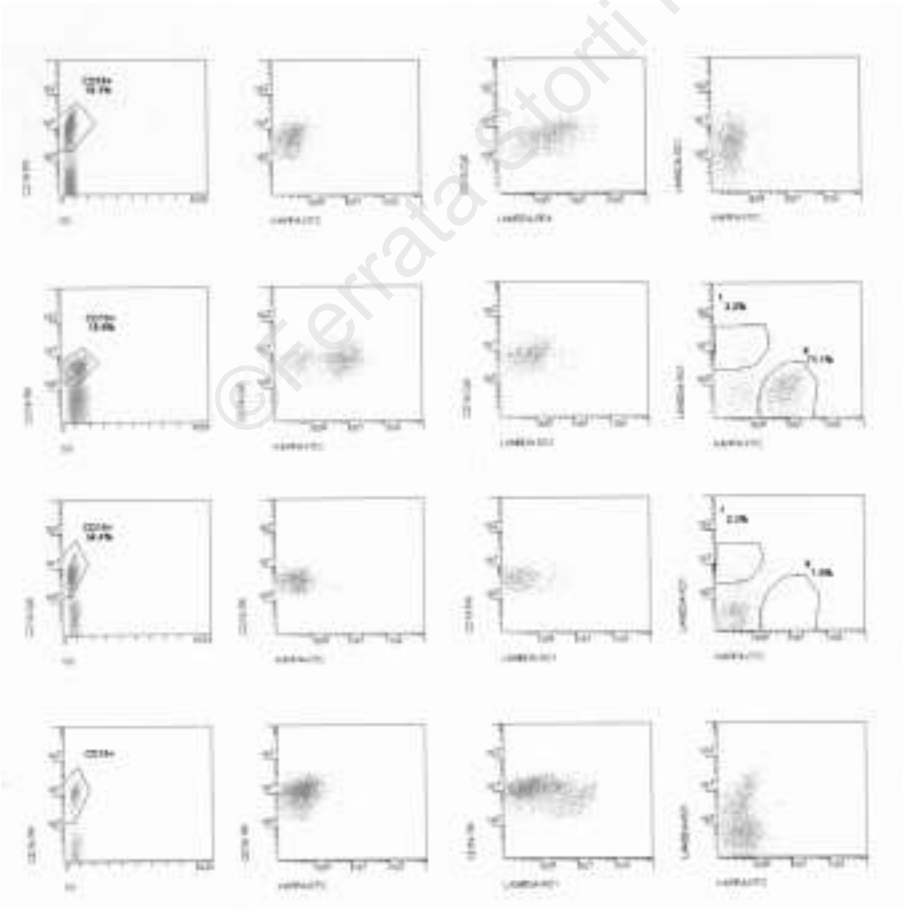

Figure 1. Flow cytometry examples of bone marrow showing lymphoma involvement. The left-hand side histograms show all lymphocytes and the selected B-cell population ($CD19^+$). In the remaining histograms we show all the κ and λ expression in the previously gated B-cell population. A: all the B-cells show clonal restriction (λ). B: abnormal κ-to-λ ratio (22:1). C: lack of expression of surface κ and λ light chains. All the B-cells are mature B-lymphocytes ($CD20^{+bright}$) (not shown). D: λ chain restriction in a subset of B-cells that express low intensity of CD19.

Gene rearrangement analysis

Sample collection and DNA isolation. Mononuclear cells were isolated from bone marrow samples by density centrifugation in a 1077 g/ mL Lymphoprep‰ (Nycomed Pharma AS, Oslo, Norway). DNA was isolated as previously described.[19]

Bcl2 (mbr). PCR amplification at the major breakpoint region (mbr) of the bcl-2/ IgH rearrangement was performed as previously described by Gribben *et al.*[20] Each sample was amplified by PCR using nested oligonucleotide primers. The original amplified product was re-amplified by a second PCR reaction using a second set of oligonucleotide primers internal to the oligonucleotides used for the initial amplification.

IGH. Amplification of the immunoglobulin gene rearrangement was performed as described by Provan *et al.*[21] Samples in which no satisfactory PCR product was obtained using the consensus primers were amplified using a semi-nested PCR.[22]

Results

Seventy-nine bone marrow biopsies from 65 patients were evaluated, 43 at diagnosis and 36 during the course of the disease. Thirty-five cases were female and there were 30 males. The pathologic diagnosis was FL on 48 occasions and DLBCL in 31. Results are summarized in Table 2.

Bone marrow morphology

The bone marrow histology showed involvement (BM^+) in 16 cases (20.3%), lack of involvement (BM^-) in 52 cases (65.8%) and 11 cases (13.9%) were undetermined or undiagnosed for involvement (BMu). The length of the trephine biopsy was considered in all cases and ranged from 0.2 cm to 4 cm (average 1.5 cm). Only 23 biopsies were longer than 2 cm. Most BMu were within the group of biopsy length inferior to 2 cm (16.1%).

Flow cytometric immmunophenotype

The FC studies were positive for involvement (FC^+) in 28 cases (35.4%) and negative in 51 cases (64.6%). In the cases with FC^+ the monoclonal B-cell population ranged from 0.3% to 88% (average 38%) of the total lymphoid gated population. There was a light sIg chain restriction in all or in a subset of the B-cell population in 22 cases (78.6%), an imbalance of the κ/λ light chain ratio was found in 3 cases (10.7%) and a lack of expression of sIg in another 3 cases (10.7%).

Within the FC^+ group, 3 cases diagnosed with FL showed a normal κ/λ ratio when all the B-cell population was gated. Despite the normal κ/λ ratio, involvement by lymphoma was established in one case due to the lack of sIg in a subset of mature B-lymphocytes and by light chain restriction in a B-cell subset with a dim expression of CD19 and high SSC in the remaining 2 cases.

There was concordance between the results of morphology and FC (BM^+/ FC^+ and BM^-/ FC^-) in 65 cases (82.3%). Concordance increased to 95.6% if the 11 BMu cases were excluded. Discrepant morphologic and immunophenotypic results (BM^-/FC^+) were found in 3 cases (3.8%). No cases showed positive morphology and negative FC (BM^+/FC^-).

All the three discrepant cases showed clonal rearrangement of bcl2-IgH and/ or IgH by PCR. Two of these cases were assessed at the initial staging. One of them was diagnosed with Ann Arbor IV-B stage DLBCL with fatal progression within the first month of diagnosis. The size of the bone marrow biopsy was 2 cm. The other case was an IE-A clinical stage FL with parotid gland involvement, and progressed seven months after diagnosis, with bone marrow and central nervous system involvement. In this case, the size of the biopsy was 1.6 cm. The percentage of monoclonal B-lymphocytes found by FC in these two cases was 26.6% (3.1% of the total celullarity) and 32% (5.6% of the total celullarity), respectively. The third case with discrepant results between bone marrow histology and FC was observed during the follow-up of a patient with FL in an apparently complete clinical remission, but with persistent bone marrow infiltration and circulating lymphomatous cells in peripheral blood assessed by FC and PCR. The histologic examination of bone marrow in this case was negative in spite of the size of the biopsy (3 cm). The percentage of monoclonal circulating B-lymphocytes was 63.3% (5.3% of the total cellularity) and all the B-cell population had sIg light chain restriction.

Among the 11 cases with BMu, 9 were FL and 2 DLBCL. All but 2 cases were FC^+. In 9 cases the results agreed with the molecular studies (PCR^+) (81.8%). One case of initial IV-A stage FL studied during follow-up was BM^+ and FC^+, but PCR negative. A second case of II-A stage DLBCL was FC- and PCR positive.

Discussion

Bone marrow biopsies are routinely performed in the staging and follow-up of patients with non-Hodgkin's lymphomas. Assessment of bone marrow involvement may affect both prognosis and therapeutic strategy, especially in DLBCL[1-7] and is also useful for evaluation of the normal bone marrow cells before therapy. The reported incidence of bone marrow involvement at diagnosis in FL and

DLBCL is 42 and 17% respectively.[11]

FC has been used extensively for the immunophenotyping evaluation of bone marrow and peripheral blood in acute and chronic leukemias,[23-26] but has not been frequently used for assessing bone marrow involvement in malignant lymphoma.

Whether FC analysis of bone marrow is useful and cost-effective in the routine staging and follow-up of patients with malignant lymphoma is uncertain. The prognostic and therapeutic implications of marrow involvement are derived exclusively from data based on morphology. A study published by Dunphy *et al.*[11] concluded that combining bone marrow morphologic examination and FC immunophenotypic data of the bone marrow aspirate yielded the most reliable interpretation of bone marrow involvement by B-non-Hodgkin's lymphoma. On the other hand Naughton *et al.*[10] concluded that FC of bone marrow aspirates has a limited role in the routine staging and follow-up of patients with malignant lymphoma. Crotly *et al.*[27] compared FC, morphology, and molecular gene rearrangements by PCR in the evaluation of bone marrow involvement and concluded that FC remains the method of choice for the detection of clonality in B-cell neoplasms, because of its higher sensitivity. The report of an international workshop to standardize response criteria for non-Hodgkin's lymphoma[8] recommends that patients whose bone marrow is histologically normal, but has a small clonal B-cell population detected by FC should be considered as having normal bone marrow until clinical studies demonstrate a different outcome for this group.

If the cases with BMu are excluded, concordance between FC and histologic examination in our study was 95.6% and only 3 discordant cases were found. These results are in agreement with others previously reported.[9,10,12,13] In all 3 cases with discrepant results, bone marrow involvement was confirmed by the molecular studies (PCR). Two of these cases were at diagnosis. One was an IE-A stage FL localized in the parotid gland. The patient was treated according to the initial clinical stage (IE-A) regardless of the flow findings, but died twelve months later due to progression of the disease. In this case the monoclonal B-cell population represented 5.6% of the total cellularity. The other case was a IV-B stage DLBCL with a wide visceral spread. In this case the presence of 3.1% monoclonal B-cells of the total cellularity could be interpreted within the context of a bulky disease that caused contamination of the bone marrow aspirate with circulating monoclonal B-cells rather than true bone marrow involvement, as suggested by some authors (Hanson *et al.*).[12] This could be a possible explanation for the discrepant results between bone marrow trephine biopsy and FC.

We feel that the FC immunophenotype has an important role in cases with BMu. Among the 11 cases in which the morphology was not diagnosed, 9 had a biopsy length < 2cm. The yield of histologically identified marrow involvement correlates with the size of the sample. International consensus recommendations of the total minimal length of the biopsy is 2.0 cm, either in unilateral or bilateral trephine biopsies,[8] though it is not always possible to obtain valuable material due to the physical and clinical conditions of the patient.

One of the main problems in FC is the lack of a guideline consensus for the study of B-cell monoclonality and the scarce information regarding FC minimal detection in lymphoproliferative diseases at diagnosis and their clinical implications. The most important indicator of B-cell neoplasms is the light chain sIg restriction. The range of normal values for the κ-to-λ ratios varies according to different authors. Some investigators recommend a predominance of one light chain over the other as indicative of monoclonal B-cell population (κ-to-λ or λ-to-κ ratios >10:1).[28] Others consider monoclonality at different λ-to-λ ratio values in accordance with their respective reference values obtained at each laboratory.[9,10,12,14] In our laboratory we established the normal κ/λ range between 1.06 and 2.05 (median 1.51) based on studies of normal bone marrow samples. As a routine we have taken between 1 and 3 as the normal range of κ/λ values compromising the sensitivity in favor of a more accurate specificity. In our study 28 cases were FC+. The monoclonal population was not observed using standard light chain analysis methods in 8 of these cases (28.6%) when the total B-cell population was analyzed. Six of these cases showed an imbalance and 2 cases had a normal κ/λ ratio. A selective gating or *clonal search* was necessary to identify the monoclonal B-cell population by using FSC and SSC versus B-cell antigen expression (CD19+ cells). Factors such as intensity of the antigen expression and cell size should be used to target neoplastic B-cell populations for λγ light chain analysis. In 3 cases the lack of expression of sλγ light chain in the mature B-cell lymphocytes was the criterion for considering FC+. All 3 cases showed clonal rearrangement of IgH genes. The expression of monoclonal antibodies such as CD20,

CD34, CD45, and TDT can help to distinguish mature from immature B-lymphocytes, which usually do not express sIg. Although technical factors should always be considered when evaluating such cases, approximately 25% of the B-cell lymphomas fail to exhibit sIg light chain expression. Kaleem *et al.*[29] described 10 well documented cases of B-cell non-Hodgkin's lymphoma that did not show sIg κ or λ light chains by dual color FC, including 3 cases of FL and 4 cases of DLBCL, therefore, loss of sIg may be used as a marker of malignancy.

We conclude that FC is both an easy and quick method for assessing bone marrow specimens in patients with lymphoma. In our hands, FC immunophenotyping is as sensitive or perhaps slightly more sensitive than bone marrow biopsy in detecting involvement by FL and DLBCL. FC study of bone marrow specimens is useful in those cases in which the morphologic evaluation of the bone marrow biopsy is not conclusive or unavailable.

Further clinical studies are necessary to establish the clinical relevance of the presence of small clonal B-cell populations in patients without histologic bone marrow involvement.

Contributions and Acknowledgments

All listed authors contributed to the conception and design of the study. CP was the principal investigator and contributed to the conception of the study, data handling and interpretation and writing of the paper. MN and CS contributed to data collection and handling. CRM performed the histologic revision. AB performed the gene rearrangement analysis. GA and AL were responsible for critical revision. We thank Montse Solsona and Ramiro Gonzalez for their daily technical work.

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

This manuscript was peer-reviewed by two external referees and by Professor Alberto Orfao, who acted as an Associate Editor. The final decision to accept this paper for the publication was taken jointly by Prof. Orfao and the Editors. Manuscript received February 20, 2001; accepted July 31, 2001.

Potential implications for clinical practice

In patients with non-Hodgkin's lymphoma immunophenotyping of bone marrow aspirates may be a useful complement of bone marrow biopsy.[30,31]

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