Analysis of surface and cytoplasmic immunoglobulin light/heavy chains by flow cytometry using a lysed-whole-blood technique: Implications for the differential diagnosis of B-cell malignancies*

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The study was aimed at the proper detection of surface and cytoplasmic clonal Ig light/heavy chains in the frame of multiparameter flow cytometry analysis of some B-cell malignancies. An exact direct evidence has been obtained that the leukemia cells following staining by antibodies to immunoglobulins will need to be washed to eliminate free plasma Igs. The results of proper Ig detection with simultaneous unaltered staining of further 2–3 markers on the cell surface after elimination of free plasma Ig in the whole blood sample are described.

In differential diagnosis of some chronic B-cell malignancies and subclassification of some acute B-leukemias the detection of intracytoplasmic light/heavy chain Igs is required. The unique phenotypic structures of multiple myeloma (MM) cells have been utilized in our approach to detect cytoplasmic Ig light and heavy chains. A modified 2-step method for analysis of cytoplasmic immunoglobulin light chains by flow cytometry in MM patients was used and the method was extended for measurement of IgM heavy chain in B-ALL. For membrane staining in MM patients' cells the combination of CD45-FITC and CD138-PE was used; the CD138 was found to be more specific than CD38 for MM cells. The whole blood cells were lysed, acquired on flow cytometry (first acquisition), then permeabilized by paraformaldehyde and saponin, and incubated with anti-kappa-FITC and anti-lambda-FITC antibodies and acquired again (second acquisition). In B-ALL patients' cells in first step the combinations of CD45-FITC or CD22-FITC and CD10-PE have been successfuly applied and after RBC lysis, acquisition and membrane permeabilization anti-IgA-FITC and anti-IgM-FITC were applied and cells were acquired again. The FITC fluorescence intensity of the second measurement was equal to the sum of surface CD45 or CD22 marker expression during the first step, and cytoplasmic clonal light or heavy chains expression during the second acquisition in both, MM and pre-B ALL patients, as well.

Key words: B-cell malignancies, flow cytometry, lysed-whole-blood technique, immunophenotyping, surface and cytoplasmic immunoglobulin light/heavy chains

Flow cytometry (FC) is nowadays the preferred method for achieving a clear discrimination between normal and neoplastic cells. The specific identification of leukemia cells by immunologic gating forms the basis for immunophenotypic diagnosis, classification and prognostic evaluation of patients with some hematological malignancies [4]. FC is useful to determine the normal counterparts of the tumor process and its differentiation state within the involved lineage [18, 27]. FC is able to detect clonality in B- or T-proliferations and criteria for malignancies by defining abnormal phenotype [7, 13, 29]. Among the different hematologic diseases, ALL can be diagnosed using FC, whereas AML is mainly confirmed by this methodology, which could moreover determine prognostic factors [27]. Immunophenotyping is also useful in chronic lymphoproliferative disorders [11], such as chronic lymphocytic leukemia using a scoring system utilizing a panel of different markers. Since FC is able to detect in multiparameter analysis simultaneously numerous cell markers, it could be more accurate than immunohistochemistry for the diagnosis of follicular lymphoma, mantle cell lymphoma, lymphoplasmacytic lymphoma/Waldenstrőm macroglobulinemia, multiple myelo-

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ma, or hairy cell leukemia [10, 14, 20-22, 28]. Finally, during the treatment follow-up, characteristic rare specific events of minimal residual disease may be detected using FC [8, 29].

There are many reasons that support the use of either whole blood or whole BM immunophenotyping techniques instead of density gradient ones. These procedures avoid selection or arbitrary loss of specific cell populations and allow reliable enumeration of the cell populations present in the sample. The direct procedure minimize the changes of modifications in antigen expression and reduce the work time. On the other hand, while whole blood/BM method is preferred, the ability to maintain a native cellular state is not always possible. The cells being stained with antibodies to immunoglobulins will need to be washed prior to antibody addition to eliminate free plasma immunoglobulins [7, 29].

In differential diagnosis of some chronic B-cell malignancies and some acute B-leukemias the detection of surface and intracytoplasmic light (L)/heavy (H) Ig chains (C) is required and forms an important part of complex phenotypic profile of B-cell malignancies in multiparameter FC analysis. CHANG et al [9] proposed 2-step method for analysis of cytoplasmic Ig light chains by FC in multiple myeloma (MM) patients. They used a 2-step acquisition procedure with fixation/permeabilization technique in between. The shifting of FITC fluorescence intensity during the second acquisition was specifically due to the cytoplasmic expression of present light chain.

The aim of our study was to show some important steps for reliable surface and cytoplasmic Ig light/heavy chains detection by FC in some B-cell malignancies using a lysed-whole-blood technique. Furthermore, an exact multiparameter approach to analyze cytoplasmic Ig light chains by 2-step acquisition technique has been shown to be extended for the study of broader spectrum of hematopoietic malignancies. The method was found to be applied also in different simultaneous marker combinations in multiple myeloma (MM) patients' cells and in exact cytoplasmic IgM heavy chain detection in pre-B ALL immunologic subtype.

Material and methods

Patients and samples. Eighty six patients with B-cell malignancies were examined in this study and their BM – less frequently simultaneously also peripheral blood (PB), lymph nodes (LN) and cerebrospinal fluid (CSF)) samples were immunophenotyped. In 8 of them BM infiltration was not proved. In 71 cases the distribution according to immune phenotype into several diagnostic categories could be performed; 7 were unclassified (Tab. 1).

Detection of cell surface antigens by FC: whole blood methods. Heparin anti-coagulated whole bone marrow/peripheral blood was incubated with fluorochrome-conjugated primary monoclonal antibodies. Cells were measured after

Table 1. Summary of clinical data, marker expression and distribution of patients with different B-cell malignancies

Diagnosis	Antigen expression	Patients No/Dg	Specimens
B-CLL	CD5+, CD23+, FMC7-, CD20dim+, CD19+, CD22+/-, monoclonal sIgdim+/negat.	26	BM, PB, LN
MCL	CD5+,CD23-, FMC7+, CD29bright+, CD19+, CD22+, monoclonal sIgbright+	3	BM, PB
HCL*	CD5-, CD11cbright+, CD22+, CD103+, CD25+, Ig (mostly)-	21	BM, PB
MM	CD45-, CD19-, CD38+, CD138bright+, cyt Ig+, monoclonal Ig mostly-	3	BM, PB
FCL	CD5-, CD23+/-, FMC7+/-, CD19+, CD20+bright, CD10bright, monoclonal mIgbright+	4	BM, PB
LPL/WM	CD5-, sIgdim+, cytIg+ (IgM), FMC7+, CD38+	3/3	BM, PB
NOT-CLASSIFIED		7	BM
Early B-ALL	HLA-DR+, CD19bright+, CD10bright+, CD20+, cyt CD22+	6	BM, PB, LN, CSF
Pre-B-ALL	HLA-DR+, CD19bright+, CD10+/-, CD20+, sIg-, cyt IgMbright	2	BM, PB, LN

*In HCL patients Ig detection (mostly negative) was not crucial for exact diagnosis; CLL – chronic lymphocytic leukemia, MCL – mantle cell lymphoma, HCL – hairy cell leukemia, MM – multiple myeloma, FCL – follicular center cell lymphoma, LPL/WM – lymphoplasmatic lymphoma/Waldenström macroglobulinemia.

Table 2. Surface monoclonal Ig light chain expression with simultaneous staining of 2 other CD markers in bone marrow cells of a patient with B-CLL

	CD5-PE + CD19-PC5, 2 x wash + K/L-FITC	2 x wash and CD19 + +CD5 + K/L at once
K/CD5	63/86 (60)*	61/86 (60)*
K/CD19	65/64 (56)*	61/55 (48)*
CD5/CD19	85/64 (60)*	86/55 (53)*
L/CD5	2/89 (1)	2/84 (1)
L/CD19	2/67 (1)	2/63 (2)
CD5/CD19	89/68 (66)*	84/63 (59)*

*cells with both markers expression.

red blood cells (RBC) lysis (Optilyse B, Immunotech, Marseille, France). Antibodies directly labeled by 4 different fluorochroms (FITC, PE, ECD and PC5) were used in 2- to 4-combinations. Cells were stained using monoclonal antibodies directed against the following surface or cytoplasmic markers (used mainly from Immunotech, Marseille, France, Binding Site, Birmingham, UK or rarely from Caltag, CA, USA) – CD3, CD4, CD5, CD7, CD8, CD34, CD38, CD71, CD138, CD10, CD19, CD20, CD22, CD45, HLA-DR, IgA, IgM, kappa, lambda, CD11b. The analysis was performed by multiparameter flow cytometry using an EPICS ALTRA flow cytometer (Beckman Coulter) equipped by Expo 32 program for analysis. The labeling procedure, analysis and membrane fixation/permeabilization are given in detail in previous studies [16, 23]. The background fluorescence level for each specimen was established using cells incubated with the appropriate fluorochrome-coupled isotype control. Whole blood sample preparation procedures usually consist in a short incubation (15 min in the dark at room temperature) of the sample with appropriate amounts of fluorochrome-conjugated MoAbs followed by RBC lysis.

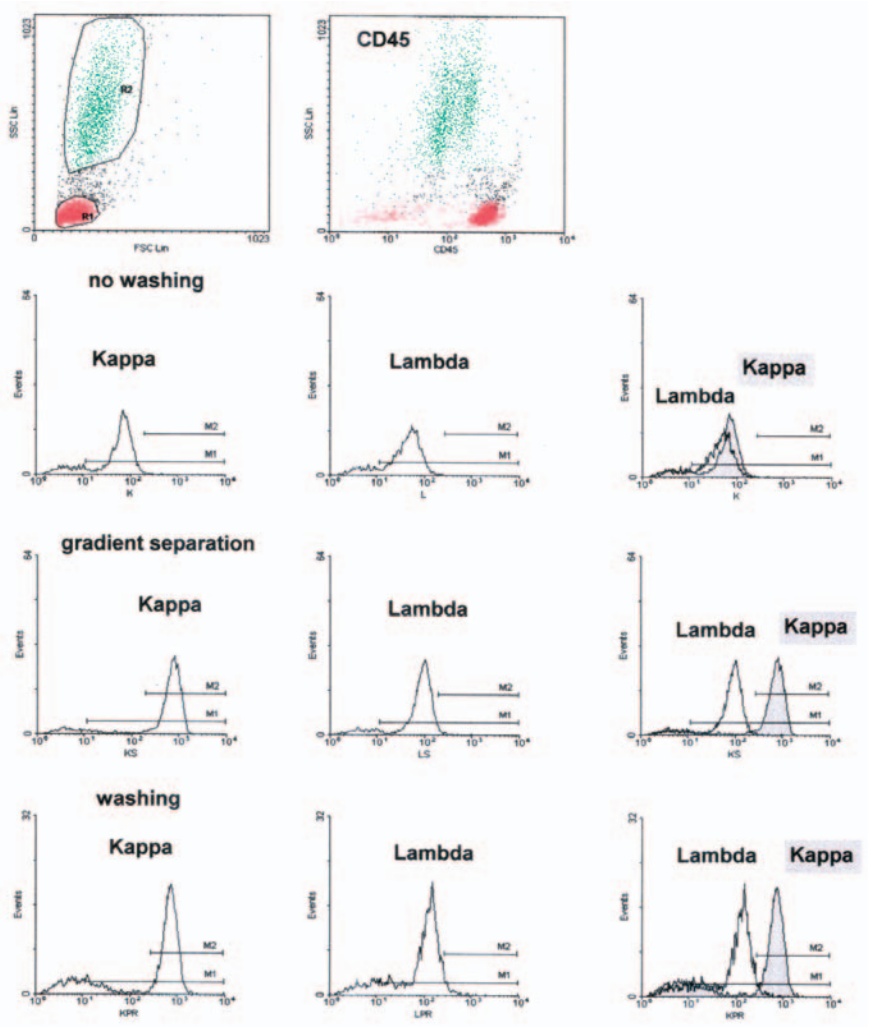

Figure 1. A representative case of bone marrow B-CLL cells; physical parameters (SSC vs FSC and SSC vs CD45) and clonal Ig light chain (kappa) expression in no washed, gradient separation obtained and double washed cells.

Analyses had to be performed on the total sample, only excluding debris/platelets, by light scatter and should be based on at least 10,000 cells per tube and ideally more than 15,000 viable cells.

Detection of surface light/heavy Ig chains by FC. Some laboratories use density gradients such as Ficoll, while others use a whole-blood-lysis methods. In specimens where density gradient samples are used, the optimal Igs detection is possible. While flow cytometry using a lysed-whole-blood technique is preferred, mainly for multiparameter FC analysis, the susceptibility of the cells to be stained with monoclonal antibodies to immunoglobulins (light and heavy chains) need to wash the cells prior to antibody addition to eliminate free plasma immunoglobulins. Contaminating serum components should be removed from the whole blood (50 μl) by washing the cells twice in 2 ml isotonic phosphate buffer solution (PBS), by centrifugation at 500 x g for 5 minutes after gentle vortex. 50 μl of packed cells is then transferred to a 5 ml tube for staining with the monoclonal antibodies. Blood cells require lysis of RBC following the staining procedure.

Detection of cytoplasmic light/heavy Ig chains by FC following fixation and permeabilization. Modified CHANG's 2-step method (2001) for analysis of cytoplasmic Ig light chains in multiple myeloma (MM) patients was used. The combinations of CD45-F (fluorescein isothiocyanate) and CD138-PE (phycoerythrin) or CD45-F/CD22-F and CD10-PE for membrane staining were applied first in MM and B-ALL patients' cells, respectively. The cells were acquired on FC (first acquisition), then fixed and permeabilized by paraformaldehyde and saponin as described before [16] and incubated with anti-kappa-F and anti-lambda-F, or anti-IgA-F and anti-IgM-F antibodies and acquired again (second acquisition). The FITC fluorescence intensity of the second measurement was equal to the sum of surface CD45 or CD22 expression during the first step and the cy-

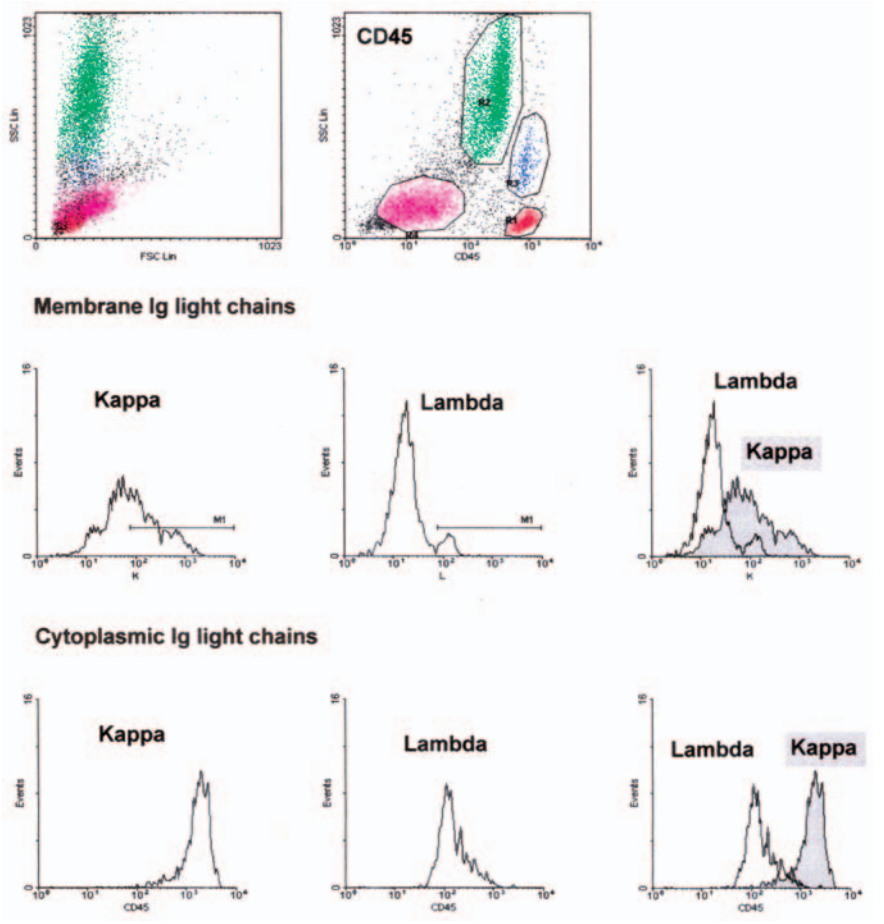

Figure 2. A representative case of bone marrow multiple myeloma cells; physical parameters (SSC vs FSC and SSC vs CD45) and simultaneous membrane and cytoplasmic clonal Ig light chain (kappa) expression (only double washed cells for Ig detection were compared).

toplasmic light chains (LC) or heavy chains (HC) expression during the second acquisition.

Results

Eighty six patients with B-cell malignancies were examined in this study and their BM (less frequently simultaneously also PB, LN and CSF) samples were immunophenotyped. In 8 of them BM infiltration was not proved. In 71 cases the distribution according to immune phenotype into several diagnostic categories could be performed (Tab. 1) on the basis of complex immune phenotype, including the clonal Ig detection; 7 cases were unclassified.

A lysed-whole-blood technique and multiparameter FC have been applied in the study of 86 patients with B-cell chronic and acute disorders. Patients' characteristics are given in Table 1. Seven of them could not be distributed into diagnostic categories. The most frequent category showed to be the B-CLL. The results showed that the Ig light and heavy chains in whole BM or PB samples were almost negative, when no sample preparation has been applied. On the other hand, the proper Ig light or heavy chains expression has been found in a majority of those samples from which the free plasma Igs were eliminated from the whole blood samples by repeated PBS washing. From 26 B-CLL the clonal Ig was detected in all but two patients. Thus, a direct evidence that the malignant cells being stained with antibodies to Igs will need to be washed to eliminate free plasma Igs has been demonstrated.

The examples of monoclonal Ig light or heavy chain values which have been the component of complex phenotype profiles and have made an important contribution to differential diagnosis of some B-cell malignancies are given in Figures 1–4 and the results of new 2-step cytoplasmic Ig light/heavy chains detection are examplified in Figures 5 and 6.

The membrane staining of BM B-CLL cells for Ig LCs after double washing procedure of whole pellet was comparable to that of density gradient one (which includes cell washing); the results of both techniques were equal and differed from that of no washing procedure, as examplified in Figure 1. In one case of the three studied multiplex myeloma (MM) patients, the monoclonal kappa LC was found to be expressed in cytoplasm and on cell surface as well; the cytoplasmic Ig LC expression is a characteristic phenotypic feature of MM cells, while simultaneous membrane expression of Ig LCs is less frequent (Fig. 2). The technique to eliminate free plasma Igs in a lysed-whole-blood sample was shown to detect IgM heavy chain in BM sample also in a patient with Waldenström macroglobulinemia, in which the percentage of pathological cells was very low (2%) (Fig. 3). The results of proper detection of membrane Igs with simultaneous unaltered staining of further 2–3 markers labeled with different fluorochromes on the cell surface after double wash of whole blood sample have been confirmed (Tab. 2, Fig. 4). The simultaneous analysis of Ig LC with other 2 markers was shown to be equal in both ways, in staining 2 surface markers first, washing the pellet and application of anti-K/L antibodies and simultaneous labeling of all markers in double washed sample, respectively. An intact marker staining

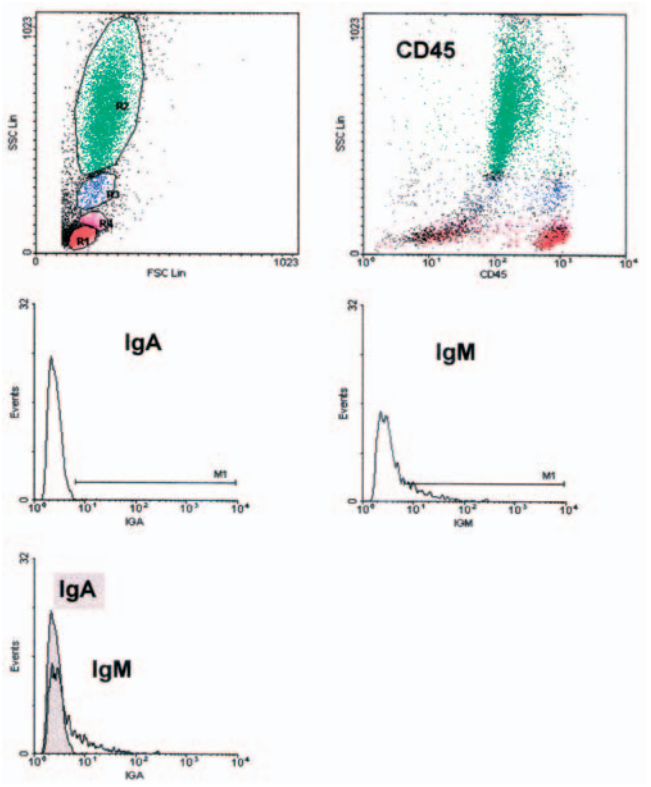

Figure 3. Arepresentative case of bone marrow Waldenström macroglobulinemia cells with as low value of pathological cells as 2%; physical parameters (SSC vs FSC and SSC vs CD45) and membrane clonal Ig heavy chain (IgM) expression (only double washed cells for Ig detection are shown).

was also found in a case when anti-CD45-ECD was added in 4-color analysis (data not shown).

To analyze cytoplasmic Ig LCs by FC in multiple myeloma (MM) patients, a 2-step method has been utilized. For membrane staining the combination of CD45-F and CD138-PE has been used. The RBC were lysed, cells acquired on FC (first acquisition), then permeabilized and incubated with anti-kappa-F and anti-lambda-F antibodies, and acquired again (second acquisition). The increase of FITC fluorescence intensity during the second acquisition is specifically due to the cytoplasmic kappa LC. Parameters are shown for both, normal lymphocyte and pathological gates, respectively (Fig. 5). In B-ALL patients' samples in first step the combinations of CD45-F or CD22-F (both with decreased expression) and CD10-PE have been applied and after RBC lysis, the acquisition and membrane permeabilization was performed and anti-IgA-F and anti-IgM-F were applied. Afterwards the samples were acquired again. The FITC fluorescence intensity of the second measurement was equal to the sum of CD45-F or CD22-F cell surface marker expression during the first step, and cytoplasmic IgM-F heavy chain expression during the second acquisition in pre-B ALL patient; both positivities could be easily separated (Fig. 6).

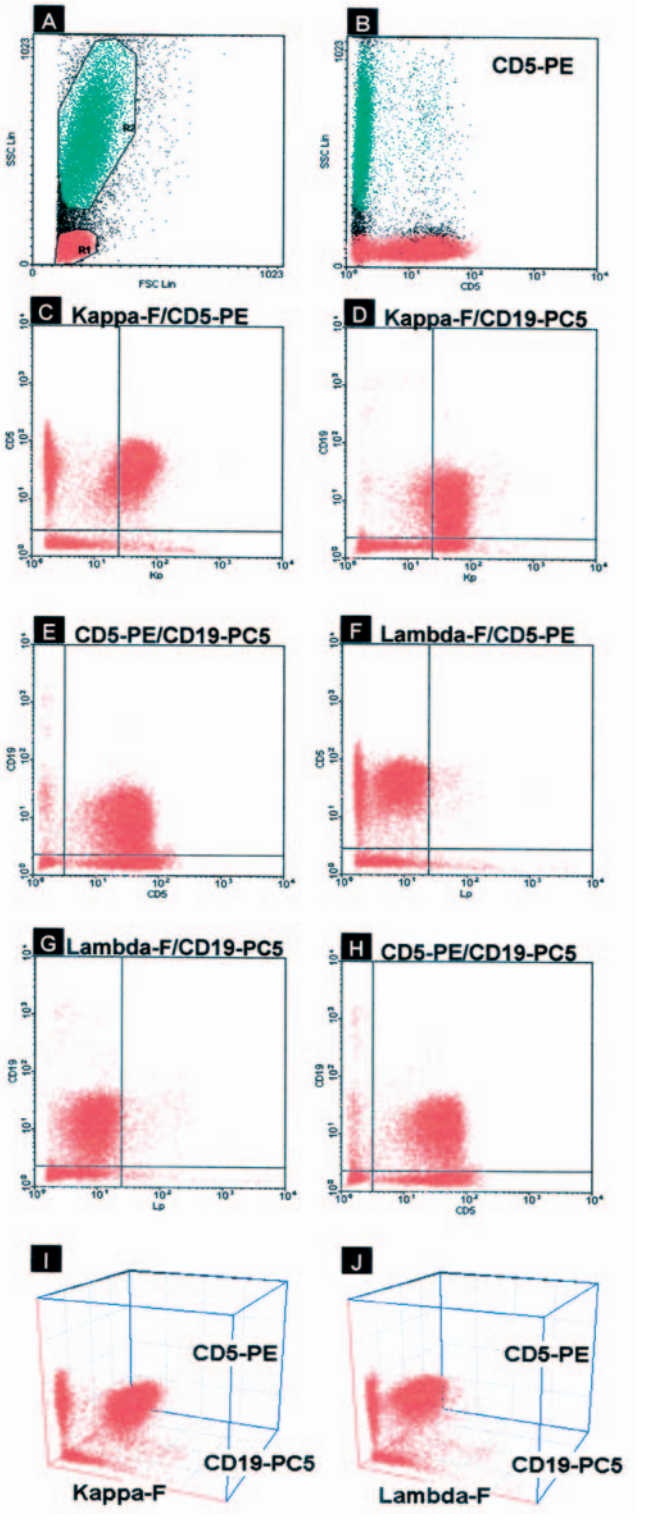

Figure 4. Simultaneous flow cytometry detection of membrane clonal Ig LC (kappa) in B-CLL patients' cells with 2 other CD markers expression (CD5 and CD19) in double washed sample prior MoAbs application in triple staining; physical parameters (SSC vs FSC and SSC vs CD45) in A,B, K/L, CD5 and CD19 expression in quadrant graphs in C–H and their 3-dimensional graphs visualization in Iand J.

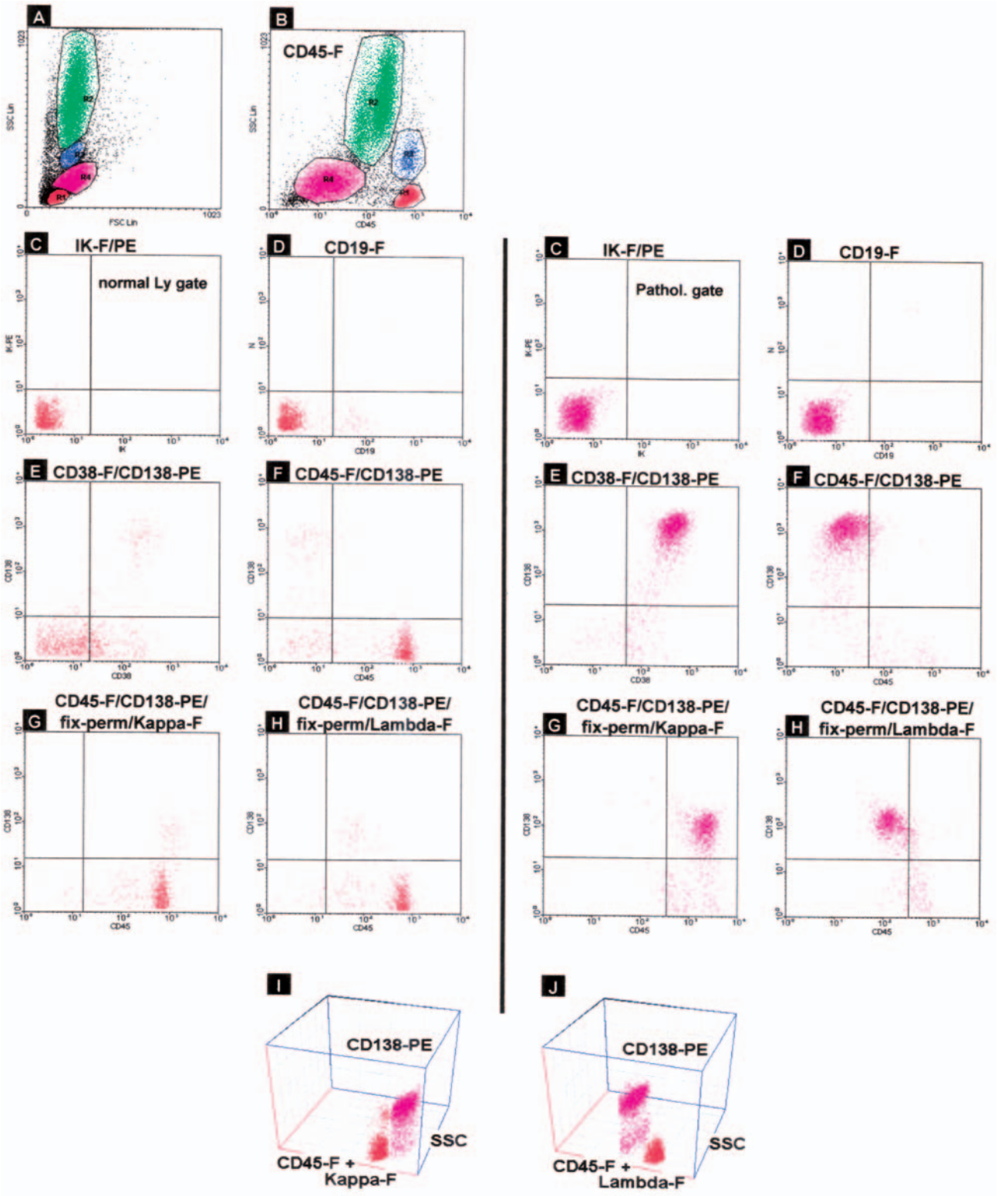

Figure 5. A representative case of 2-step cytoplasmic flow cytometry Ig light chain detection in multiple myeloma BM patients' cells. Physical parameters (SSC vs FSC and SSC vs CD45) of measured sample are shown in A and B, isotype negative control and characteristic surface markers detected after RBC lysis in the first acquisition in C–F. After fixation/permeabilization anti-lambda and anti-kappa antibodies were applied to cell pellet and acquired again. FITC flores-cence of both, CD45 and kappa/lambda LC is shown in G and H. Results are given for both, normal (left column) and pathological (wright column) gate, respectively. 3-dimensional graphs common for both, normal and pathological populations, are shown in I and J.

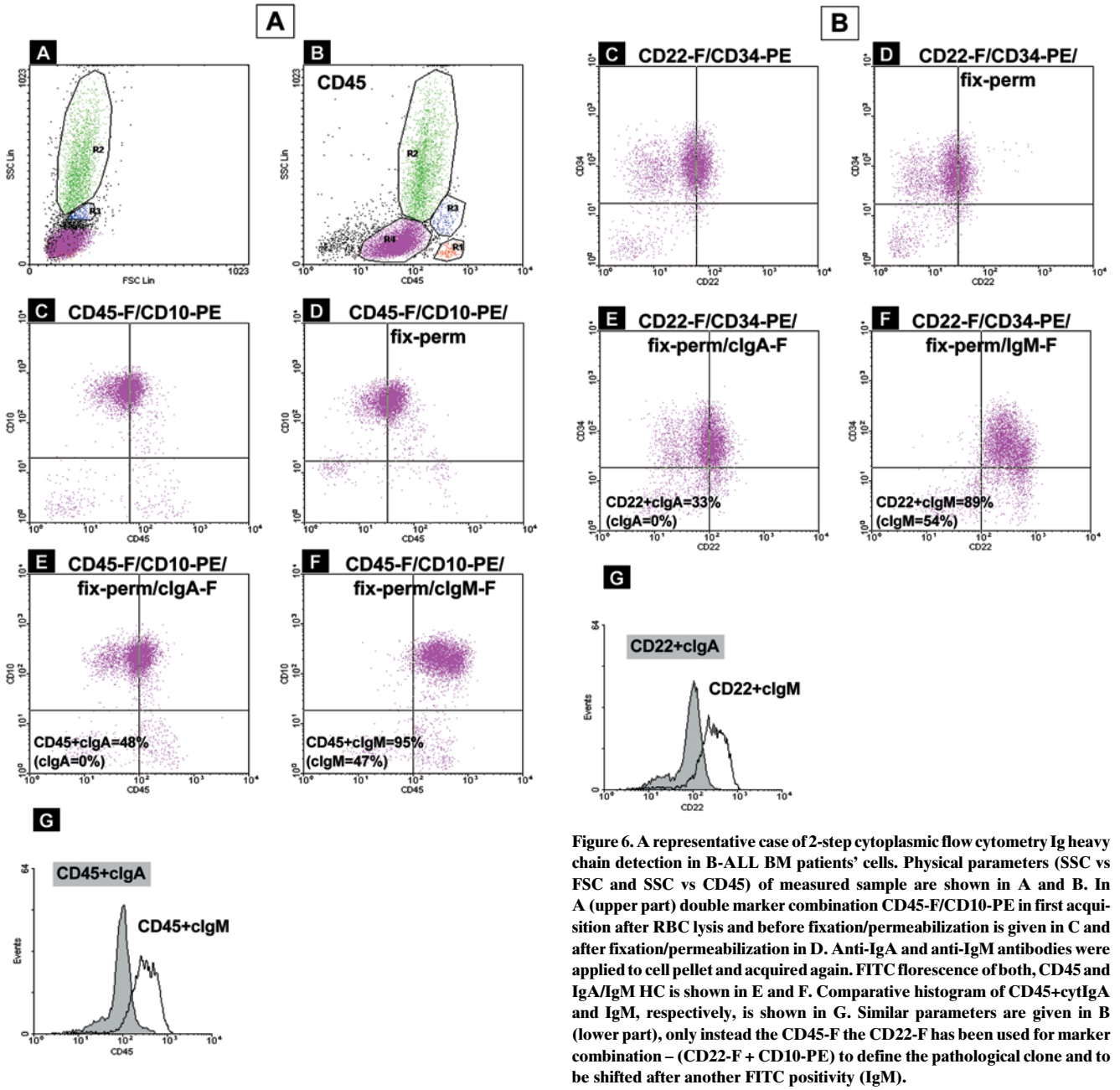

Figure 6. A representative case of 2-step cytoplasmic flow cytometry Ig heavy chain detection in B-ALL BM patients' cells. Physical parameters (SSC vs FSC and SSC vs CD45) of measured sample are shown in A and B. In A (upper part) double marker combination CD45-F/CD10-PE in first acquisition after RBC lysis and before fixation/permeabilization is given in C and after fixation/permeabilization in D. Anti-IgA and anti-IgM antibodies were applied to cell pellet and acquired again. FITC florescence of both, CD45 and IgA/IgM HC is shown in E and F. Comparative histogram of CD45+cytIgA and IgM, respectively, is shown in G. Similar parameters are given in B (lower part), only instead the CD45-F the CD22-F has been used for marker combination – (CD22-F + CD10-PE) to define the pathological clone and to be shifted after another FITC positivity (IgM).

Discussion

Chronic lymphoid leukemias comprise a number of biologically distinct neoplasms of mature lymphocytes. In most cases, detailed immunophenotypic analysis of the CLLs permits specific classification and initiation of the most appropriate therapy [11]. For example, the characteristic immunophenotype of *B-CLL* – CD5+, CD23+, FMC7-, CD20dim+, clonal sIgdim+/negat, distiguishes it from another CD5+ B-cell lymphoproliferative disorder, mantle cell lymphoma, which typically displays the composite phenotype – CD5+, CD23-, FMC7+, CD20bright+, clonal sIgbright+. Likewise, hairy cell leukemia has a characteristic immunophenotype – CD5-, CD11c bright+, CD22+, CD103+, CD25+, as described in previous paper [2], which distinguishes it from other CD5- B-cell lymphoproliferative disorders, including the multiple myeloma, CD45-, CD19-, CD38+, CD138+ , cytIg+. Furthermore, normal and malignant circulating plasma cells (PC) can reliably be identified using anti CD138/CD38 and CD45 MoAbs. CD45 expression separates PB-PCs into two subsets of which the CD45- one only occurs in myeloma subsets [31]. These unique

phenotypic structures of MM cells have been utilized in our approach to detect cytoplasmic Ig light and heavy chains. SIgs are traditionally considered to be absent in PC. However, up to one third of myeloma patients PC are positive for sIgs as well [25], as was found in one out of our 3 patients. SAN MIGUEL et al [27] recognized that in MM at diagnosis almost all BM-PCs (>95%) are phenotypically aberrant that forms a very good basis for MRD detection. ISHIKAWA et al [19] found that malignant plasma cells (myeloma cells) isolated from MM patients lack the CD19 expression, in line with our results, and they suggested that the loss of CD19 could contribute to the proliferative advantage of the malignant plasma cell clones in this disease.

Immunophenotypic analysis by FC may also identify clinically relevant subsets of patients within a diagnostic category of leukemia. Thus, in patients with B-ALL, the subset with worse prognosis and high risk therapy could be separated (pre-B, cytIgM+ phenotype) [5], or in CLL, in which the deviation from the typical immunophenotype, caused also by sIg expression, is associated with trisomy 12 and mixed-cell morphology [11].

Our results were aimed at exact detection of surface and cytoplasmic Ig light/heavy chains in the frame of multiparameter FC analysis of chronic B-cell malignancies. The results of proper detection of sIgs (Fig. 1–3) with confirmation of simultaneous unaltered staining of further 2–3 markers labeled with different fluorochromes (Tab. 2, Fig. 4) after double wash of the whole blood sample have been shown. In differential diagnosis of some chronic B-cell malignancies and subclassification of some acute B-leukemias, the detection of intracytoplasmic light/heavy chain Igs is required. A 2-step acquisition procedure with fixation/permeabilization technique in between has been utilized. We have modified the method of CHANG et al [9] for study of MM patients' cells and extended the method for measurement of cytIgM heavy chain in B-ALL. For the membrane staining in MM patients we used the combination of CD45-F and CD138-PE (Fig. 5), as we found the CD138 to be more specific than CD38 for MM diagnosis. The specificity of the 2-step method for clonal Ig kappa LC detection is demonstrated in Figure 5, where results are given for normal lymphocyte gate, as well. In B-ALL patients' samples in the first step the combinations of CD45-F or CD22-F (both of lower expression in leukemia cells) and CD10-PE have been applied. We have shown, that CD22-F in combination with other surface CD marker (CD10-PE) could be used equally well as CD45-F + CD10-PE in cyt L/H chain 2-step acquisition technique (Fig. 6). FITC fluorescence intensity of the second measurement was equal to the sum of surface CD45 or CD22 marker expression during the first step, and the cytoplasmic L or H chains expression during the second acquisition in MM (Fig. 5) and pre-B ALL (Fig. 6), respectively.

Immune phenotyping has become one of the essential methods for proper classification of lymphoid neoplasms and has added a new dimension to the diagnosis of lymphoma [1, 3, 17, 18, 30]. A number of monoclonal antibodies are available to recognize various epitopes based on the cell surface or cytoplasm [6, 12, 15, 24, 26]. It follows from the study, that leukemia/lymphoma cells cannot be classified with a single marker. Instead, the use of a monoclonal antibody panel consisting of multiple antibodies is required to support the provisional diagnosis based on morphological findings.

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