High prognostic value of measurable residual disease detection by flow cytometry in chronic lymphocytic leukemia patients treated with front-line fludarabine, cyclophosphamide, and rituximab, followed by three years of rituximab maintenance

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Supplementary Material

Flow Cytometry

At diagnosis all samples were collected in tubes containing K3 EDTA as anticoagulant. BM samples were immediately diluted 1/1 (vol/vol) in phosphate-buffered saline (PBS). Whole BM and PB samples (approximately 2x10^6 cells in 100ml per test) were stained and lysed using a direct immunofluorescence technique. The following staining – fluorescein isothiocyanate (FITC)/phycoerythrin (PE)/PE-Cyanin 5 (PE/Cy5)/Allocycoyianin (APC) were systematically used: 1) CD22/CD23/CD19/CD5; 2) FMC7/CD43/CD19/CD5; 3) CD103/CD25/CD19/CD5; 4) CD10/CD11c/CD19/CD5; 5) CD20/CD38/CD19/CD5; 6) CD81/CD22/CD19/CD5; 7) CD20/CD49d/CD19/CD5; 8) sIgk/sIgl/CD19/CD5, and 9) ZAP70/CD3+CD56/CD19/CD5. The source of all monoclonal antibodies except ZAP70 was Becton Dickinson Biosciences (San José, California, USA). ZAP70 was purchased from Immunotech (Marseille, France). Before staining, the polyclonal sIgKappa/sIgLambda/CD19/CD5 tests were washed twice (5 min at 540 g twice) in 4ml of PBS in order to remove serum Ig. For staining purposes, samples were incubated for 15 min (at room temperature in the darkness) in the presence of the above mentioned reagents.

Afterwards, 2 ml of FACS lysing solution (Becton Dickinson Biosciences) diluted 1/10 (vol/vol) in distilled water was added to each tube and after gentle vortex, samples were incubated for another 10 min under the same conditions as those mentioned above, in order to lyse the non-nucleated red cells. Then, cells were centrifuged (5 min at 540 g) and the cell pellet washed in 4ml of PBS. Finally, cells were resuspended in 0.5 ml of PBS until measured in a FACSCalibur (Becton Dickinson Biosciences) equipped with two lasers, an air-cooled argon laser and a red diode laser using the CellQuest software programme (Becton Dickinson Biosciences). A total of 20,000 events, corresponding to the total nucleated cells present in the sample were acquired. Light scatter signals were recorded in linear scale and fluorescence signals in logarithmic mode. For data analysis, the Paint-A-Gate PRO software (Becton Dickinson Biosciences) was used. B lymphocytes were identified according to their SSC/CD19+

distribution and their relative numbers were calculated after excluding cell debris and platelets. In addition, for each antigen expressed on B cells its mean fluorescence intensity (MFI) expressed in fluorescence channels (arbitrary relative linear units scaled from 0 to 104) was recorded for pathologic B cells. Antigen expression was then classified as being negative (−), dimly positive (+d), positive (+) and strongly positive (++) using arbitrary relative linear MFI values of 0 to 5, 5 to 10^1, 10^1 to 10^2 and more than 10^2, respectively. We expressed the total percentage of pathological B cells CD38 and CD49d positive respectively.

Measurable residual disease

Samples were prepared as previously described. The analysis of the measurable residual disease was performed, in samples from peripheral blood and bone marrow after induction and from bone marrow during Rituximab maintenance, with a combination of monoclonal antibodies slightly modified from the ERIC protocol: 1) CD20/CD38/CD19/CD5; 2) CD81/CD22/CD19/CD5; 3) sIgL/sIgK/CD19/CD5; and 4) CD22/CD79b/CD19/CD5. CD43 was not included in the analysis: we included the last combination instead of CD43/CD79b/CD19/CD5 based on our previous experience with that combination in the analysis of MRD in CLL. The minimum number of pathological B cells acquired was performed according to the ERIC recommendations. Acquisition was performed in two consecutive steps in order to increase the sensitivity of the analysis. First a total of 20,000 events corresponding to the total nucleated cells were acquired. In the second step, acquisition through a "live gate" drawn on the SSC/CD19+ region in which B lymphocytes are located was performed. To achieve a limit of detection of 0.01% we acquired a minimum of 200,000 events if the minimum population size is 20 and 500,000 events if the minimum population size is 50.

ZAP70 method:

Fresh blood samples were obtained on EDTA (analysis was performed in the same day the sample arrived to the laboratory). We used SB-ZAP70-FITC (Immunotech, Marseille) with the FIX&PERM (Caltag laboratories) kit for the fixation and permeabilization steps. The percentage of ZAP70 B-CLL cells was expressed according to ZAP70 expression in T-lymphocytes (CD3+ lymphocytes). ZAP70 was considered positive when more than 20% of B-CLL cells expressed this marker.