National Standardization of ZAP-70 Determination by Flow Cytometry: The French Experience

Magali Le Garff-Tavernier, Michel Ticchioni, Martine Brissard, Céline Salmon, Sophie Raynaud, Frédéric Davi, Alain Bernard, Hélène Merle-Béral, Florence Ajchenbaum-Cymbalista, and Rémi Letestu

1Hematology Biology Department, CHU Pitié Salpêtrière, Paris, France
2Immunology Biology Department, Hôpital de l’Arche, CHU Nice, Nice, France
3Hematology Biology Department, CHU Avicenne, Bobigny, France
4Hematology Biology Department, Hôpital Pasteur, CHU Nice, Nice, France

Background: ZAP-70, after being considered as a potential surrogate for VH mutational status, has seen its own prognostic value emerge. We aimed at standardizing a simple, fast, and reproducible flow cytometry method.

Methods: AntiZAP-70 antibody 2F3.2 was used with indirect labeling and secondary anti-IgG2a antibody. The reference values for the expression of the results were determined on 45 normal blood samples. ZAP-70 protein expression was investigated in 192 CLL samples. The indirect technique was compared with FITC-conjugated 2F3.2 clone, and with clone 1E7.2-FITC, -PE or -AlexaFluor 488.

Results: Using FITC or PE-conjugated antibodies, 2F3.2 and 1E7.2 clones allowed a much less adequate discrimination between positive and negative cells and discordant cases were most likely true negative cases. Using the AlexaFluor 488 conjugated 1E7.2 clone, the discordant cases were mostly negative with the conjugated antibody and positive with the 2F3.2 clone but Western blotting or RNA microarray confirmed discordant cases were false negative with the conjugated antibody. Subsequently, recommendations were used by 13 centers participating in an interlaboratory quality control protocol. The use of MFI ratio appeared to be more reliable.

Conclusions: Results suggested that slight differences in the procedure had little impact on the interpretation in characteristic cases; however, careful interpretation was required for values close to threshold.

Key terms: ZAP-70; flow cytometry; CLL; harmonization
Different techniques may be used to explore ZAP-70 expression in CLL cells: at the DNA level by studying promoter methylation (8), at the RNA level using PCR methods (9,10), or at the protein level (4,5,11). As different lymphocyte subsets normally express ZAP-70 (12), the technique used has to discriminate between ZAP-70 expression in CLL cells and physiologic expression. Most techniques are sensitive to the presence of T or NK cells. PCR and Western blot (WB) require a selection of leukemic cells whereas methylation study tolerates a higher level of contamination. Therefore, flow cytometry appeared a promising method allowing to measure ZAP-70 expression in different lymphocytes subsets defined on the basis of their expression of selected membrane markers. In this collaborative work, we aimed at developing a simple, fast, and reproducible flow cytometry approach for ZAP-70 determination in CLL.

PATIENTS AND METHODS

Cell Samples

Three institutions participated in the study and 13 centers were involved in interlaboratory quality control. Peripheral blood was obtained from 192 untreated CLL patients followed in these centers and from 45 normal subjects after informed consent.

Purification of B Cells

Purification of B cells was performed by either negative selection using RosetteSep (ref. no.15024, StemCell Technologies, Grenoble, France) or positive selection on CD19 expression using LS column (ref. no. 130-042-401, Myltenyi Biotec, Paris, France). The two techniques gave comparable results in terms of purity of the sample (96-98% of CD19 positive cells were collected). The RosetteSep technique is easy, fast, does not require columns and magnets, and could be run in parallel with Ficoll. The drawback is the necessity to use fresh whole blood samples with the presence of red blood cells, making it unsuitable for conserved frozen samples.

Western Blot

Western blotting was conducted on purified B cells. Briefly, after standard lysis, migration on polyacrylamide gel and transfer on nitrocellulose membrane, the 2F3.2 clone (Upstate) anti-ZAP-70 antibody was used at 1/1000e dilution. ZAP-70 protein was revealed using a rat antimouse HRP-conjugated secondary antibody and ECLTM Western blotting detection reagent (ref. no. RPN2209, Amersham-Biosciences, Saclay, France) as substrate.

Flow Cytometry

Determination of ZAP-70 expression was performed either on freshly isolated mononuclear cells by Ficoll-Hypaque density gradient or on DMSO frozen cells. Different procedures were described with regard to ZAP-70 protein labeling with respect to consensual minimal recommendations. In an attempt at homogeneity, the use of a commercial kit for fixation and permeabilization was recommended. The Fix and perm kit from Caltag was selected.

As the equipment was different from one center to another, the choice of the fluorochrome remained open. In contrast, the membrane markers were imposed including antibodies directed against CD19 as B-cell marker, CD3 and CD56. The addition of an anti-CD5 antibody was recommended to discriminate between residual normal B-lymphocytes and CLL cells. This combination was supposed to allow the discrimination of B-CLL cells, residual T lymphocytes and NK cells, and normal residual B cells.

Two different clones of anti-ZAP-70 antibodies were tested. The following purified or directly conjugated antibodies were purchased from different manufacturers: FITC conjugated clone 2F3.2 (Upstate); PE conjugated clone 1E7.2 (eBioscience); PE conjugated clone 1E7.2 (Tebu Caltag); and Alexa 488 conjugated clone 1E7.2 (Tebu Caltag). When purified 2F3.2 clone was used, the secondary antibody was FITC conjugated and different reagents were tested as describe below.

The mode of expression of results is crucial for correct interpretation of the analysis. We evaluated thresholds related to isotypic control and to ZAP-70 expression in T cells as determined by Crespo et al. In order to include an internal control we considered mean fluorescence intensity (MFI) in T lymphocytes (Tly) and CLL cells. Cut-offs were defined as described in Table 1. It was recommended to acquire on the flow cytometer at least 1,000 Tly, and as many CD5 negative B-lymphocytes (Bly) as possible.

Interlaboratory Quality Control Scheme

The recommendations described above were evaluated in 13 centers participating in CLL studies. For the purpose of standardization, the use of commercial reagents for fixation and permeabilization was decided. For ZAP-70 labeling, as we obtained discordant results with conjugated anti ZAP-70 antibodies, the use of indirect technique was preferred. The FITC conjugated secondary antibody could be either anti-IgG or specific anti-IgG2a. Four different CML samples were selected on the basis of their characteristic pattern of ZAP-70 expression. DMSO frozen cells were sent to the participating centers in dry ice and processed according to the minimal recommendations. Results were reported according to two guidelines: percentage of CLL cells above a threshold set on the expression of ZAP-70 on T lymphocytes (%/Tly) and a MFI ratio = MFI ZAP-70 T lymphocytes/MFI ZAP-70 CLL cells (Tly/CLL ratio).

Table 1

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
<th>Positivity threshold</th>
<th>Inconclusive results</th>
</tr>
</thead>
<tbody>
<tr>
<td>%/T-Ly</td>
<td>% of Zap-70 positive B-CLL cells according to ZAP-70 expression in T-Ly</td>
<td>≥20%</td>
<td></td>
</tr>
<tr>
<td>T-Ly/CLL</td>
<td>ZAP-70 mean fluorescence intensity T-Ly/B-CLL ratio</td>
<td>&lt;4</td>
<td>4-5</td>
</tr>
</tbody>
</table>

Cytometry Part B: Clinical Cytometry DOI 10.1002/cyto.b
RESULTS

The expression of ZAP-70 protein was investigated in 192 samples of CLL patients from three different institutions.

Validation of Flow Cytometry Results by Western Blotting

We compared results obtained by WB on purified B cells with those obtained by flow cytometry on 50 cases and a good concordance was observed (Fig. 1). Twenty-one cases were found ZAP-70 negative by either WB or cytometry; 23 were ZAP-70 positive by both techniques. None was found positive by cytometry and negative by WB, but 6 cases appeared positive by WB only, and considered negative in cytometry. Positivity of WB in these cases might be interpreted as contamination by T cells in the purified B cell extracts used for WB.

Choice of Secondary Antibody

ZAP-70 expression was compared in 21 CLL patients and one normal B cell control by indirect labeling with either anti-IgG or anti-IgG2a secondary antibody. There was a good correlation between the results obtained with either one and no discordant result was observed among the 4 negative and 17 positive cases studied (Fig. 2). Therefore, both antibodies are suitable, but the specificity of anti-IgG2a is of interest to alleviate the staining protocol and to avoid the risk of losing formalin sensitive antigens.

Threshold of Positivity Was Determined on a Series of Normal Blood Samples

Using a specific anti-IgG2a secondary antibody, we investigated a series of 45 normal blood samples. We aimed at establishing reference values for the different modes of expression of the results (Table 1). The use of a threshold related to isotypic control was clearly not optimal. As the results were highly variable from one sample to another (CV = 93%), this mode of expression was not further considered. We considered a threshold relative to T-Ly as previously described for CLL. Interestingly, although variable among different healthy volunteers, a threshold relative to ZAP-70 expression in T cells at 20% for positivity as determined by Crespo et al. (4) was applicable to normal blood samples. As we observed a significant variability of ZAP-70 analysis, we also evaluated the use of MFI ratio as a new mode of expression of the results. Considering the T-Ly/B-Ly MFI ratio, the results were similar whether normal B-lymphocytes were CD5+ or CD5−. Based on the mean of T-Ly/normal B-Ly ratio, a positivity threshold at 4 was established.

Choice of the Anti ZAP-70 Antibody

Comparison of purified versus FITC conjugated 2F3.2 clone (Upstate). Eighty-three patients have been tested with both techniques. The results were discordant in up to 36% (30/83 pts) of cases, mostly because cases that were negative by the indirect technique appeared positive with the conjugated antibody. Figure 3 (panels A and B) illustrates the discrepancy between both techniques. Using the conjugated antibody allowed a much less adequate

<table>
<thead>
<tr>
<th>FCM</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZAP-70 actin</td>
<td>31%</td>
<td>19%</td>
<td>&lt;1%</td>
<td>&lt;1%</td>
</tr>
</tbody>
</table>

Fig. 1. Western blot analysis. Comparison of flow cytometry and western blot data in four representative cases. In the case 1, Western blot was interpreted as a carryover of the extract by residual T lymphocytes.

Fig. 2. Choice of the secondary antibody. ZAP-70 expression was studied in 22 samples using either anti-IgG or specific anti-IgG2a secondary antibodies. There was a good coefficient of correlation between the two techniques independent of the mode of expression of the results used. Panel A shows results as % relative to T-Ly and panel B as T-Ly/B-CLL ZAP-70 MFI ratio.
discrimination between positive and negative cells. Moreover, the distribution of T cells was clearly overlapping on negative cells in both negative CLL cases and normal blood samples. Therefore, we concluded that the discordant cases were most likely true negative cases that appeared mistakenly positive with conjugated antibody.

Comparison of purified 2F3.2 clone with fluorochrome conjugated 1E7.2 clone. Two other commercially available conjugated antibodies derived from the 1E7.2 clone have also been tested. These antibodies were either PE-conjugated (eBioscience and Caltag) or Alexa-Fluor 488-conjugated (Caltag).

PE-conjugated 1E7.2 was tested in 54 cases and compared with 2F3.2 clone using indirect labeling. On the 9 cases tested simultaneously with both PE-conjugated antibodies, we obtained similar results with a heterogeneous labeling on the T cell population that was even more divergent with the eBioscience antibody. Therefore, only Caltag antibody was used for further comparison. We observed 17/54 discordant cases (31%) among which in most cases ZAP-70 expression was interpreted as negative with 2F3.2 clone and positive with PE-conjugated 1E7.2 antibody. The analysis of the graphs led to a similar conclusion as in the previous experiment with FITC conjugated 2F3.2 clone. Besides a decrease in ZAP-70 fluorescence intensity, we suspected that the heterogeneous labeling of the T cells might also hamper the determination of the position of the positivity threshold (Fig. 3, panels A and C). This pattern of ZAP-70 expression being observed in normal blood samples as well, we concluded that most of the discordant cases were misinterpreted as false positive.

Finally we compared purified 2F3.2 clone with Alexa488-conjugated 1E7.2 antibody in 56 independent samples. In contrast with the previous directly conjugated antibody tested, the discordant cases were mostly negative with the conjugated antibody and positive with the 2F3.2 clone. It was not possible to reinvestigate all the 14 discordant cases but examination of 6/14 by either WB or RNA microarray confirmed the presence of ZAP-70 expression. Therefore these cases were interpreted as false negative.

Technical Consensus and Validation

The results of the different experiments allowed establishment of minimal recommendations for ZAP-70 evaluation by flow cytometry that were evaluated in 13 centers participating in a national multicentric protocol. This study was designed as an interlaboratory quality control. Although not strictly identical from one center to another, the procedures used allowed the 13 participating centers to give the same interpretations in 3 samples out of 4 (1 positive and 2 negative). In all cases, %/T-Ly showed a higher coefficient of variation as compared with T-Ly/CLL ratio. The fourth sample was selected for the difficulty to draw a conclusion on its level of ZAP-70 expression. As expected, discordant interpretations were reported. The results were deduced as negative in 5 centers (ranging from 11 to 19% of positive cells relative to T-Ly) and positive (ranging from 30 to 92% of positive cells relative to T-Ly) in the 8 remaining centers. The sample was tested using an alternative method and confirmed negative.

As this variation might be the consequence of the freezing procedure, we analyzed the impact of cryopreservation on ZAP-70 detection. We compared fresh and frozen samples from 17 CLL patients (5 positive, 12 negative) and 3 normal controls. In normal and patient samples, the conclusions on ZAP-70 status were identical before and after cryopreservation. These preliminary data need to be confirmed on a larger series of samples to determine if the positivity threshold should be refined depending on the type of sample (fresh or frozen) investigated.

DISCUSSION

The aim of the first step of this study was to validate ZAP-70 quantification technique. At the beginning of our study, only one flow cytometry method had been published (4).
based on the use of 2F3.2 anti-ZAP-70 antibody and indirect labeling. First we aimed at validating this approach in our hands. For this purpose, we decided to systematically compare results obtained by Western blot on purified B cells with those obtained by flow cytometry. The concordance was good with only 6 cases appearing positive by WB and negative in cytometry. These results were interpreted as contamination by T cells in the purified B cell extracts used for Western blot.

Depending on the antigens targeted and the antibodies used, the detection of B-cell markers after fixation procedure may be impaired. However, as the indirect technique was preferred, intracytoplasmic staining was supposed to precede membrane labeling. In order to simplify the protocol, the use of a specific anti-IgG2a secondary antibody allowed membrane staining prior to fixation provided only antibodies of IgG1 isotype are used at this step are tested and found of interest to simplify the staining protocol.

We then aimed at establishing reference values for the different modes of expression of the results in a series of 45 normal blood samples. Interestingly, a threshold relative to ZAP-70 expression in T cells at 20% as determined by Crespo et al. (4) was applicable to these normal blood samples. In order to include an internal control in the interpretation of the results and to minimize the inter-operator variability in subsequent multicentric applications, we also considered the use of MFI ratio for subsequent studies.

As intracellular labeling using an indirect method is time consuming, when available, fluorochrome conjugated antibodies are more adapted to routine use. Therefore, the indirect technique was compared with different directly conjugated antibodies. Using FITC-conjugated 2F3.2 and PE-conjugated 1E7.2 antibodies allowed a much less adequate discrimination between positive and negative cells with a distribution of T cells clearly overlapping on negative cells in both negative CLL cases. Therefore, it seems that the ~30% discordant cases we found were most likely true negative cases that appeared mistakenly positive with the conjugated antibody. This conclusion was reinforced by the observation of similar results when the conjugated antibodies were tested on normal blood samples. Either the size of the PE fluorochrome or the particular subcellular localization of ZAP-70 protein could be involved in the impaired staining observed with conjugated antibodies. In any case, a problem with fluorochrome conjugation cannot be ruled out.

In contrast, using the Alexa 488 conjugated 1E7.2 clone, the discordant cases were mostly negative with the conjugated antibody and positive with the 2F3.2 clone. WB or RNA microarray confirmed that most of these latter discordant cases should be considered as false negative with the conjugated antibody. These results raised the question of the sensitivity of this antibody. Moreover, the immunogenic peptide used to raise 1E7.2 clone spans the tyrosine Y292, which is an important functional site. However little is known regarding the impact of Y292 phosphorylation and Cbl interactions on 1E7.2 binding ability in CLL cells.

Minimal recommendations from this preliminary study were subsequently used in 13 participating centers involved in CLL studies. A multicentric interlaboratory quality control was organized, which raised different issues and comments. Overall, attempts to standardize procedures gave encouraging results since no discordant conclusions were observed in patients with a characteristic pattern of ZAP-70 expression. When interpretation is more complex, additional interpretation guidelines (such as combining percentage and ratio) might be required. However, it should be kept in mind that distribution of ZAP-70 levels of expression follows a continuum and that binary interpretation (i.e. positive or negative) might be inappropriate. The use of MFI ratio appeared less variable as compared with percentage of positive cells but required also to establish normal values on normal control samples. Whether combining both methods improves the standardization is currently under evaluation on a larger series of patients. In all cases we recommend providing the physician with both the raw data and an interpretation of the analysis.

In conclusion, in this study, as part of a French National Protocol proposed by the Health Department of the Government, we reached a technical consensus on different features of the determination of ZAP-70 expression by flow cytometry. Pre-analytical, analytical and interpretation issues were considered and led to recommendations for standardization of the technique. Our method was also tested and validated in a multicentric trial. The results suggested that slight differences in the procedures have little impact on the interpretation in characteristic cases. However, careful interpretation is required for values close to the threshold.

ACKNOWLEDGMENTS

We acknowledge Drs. Christine Arnoulet, Lucile Baseglio, Michel Degenne, Franck Genevieve, Marie-Christine Jacob, Marc Maynadie, Jean-Yves Perrot, Anne Ponquet Stéphanie Poulain, and Véronique Salain for their participation in the interlaboratory quality control. We are grateful to Tebu-Caltag, eBioscience, and Upstate laboratories for providing us with some of the reagents used in this study.

LITERATURE CITED


