INTRODUCTION: Clonality of a B cell expansion is usually the basis for the diagnosis of B-cell chronic lymphoproliferative disorders (B-CLPDs). Nevertheless only considering light chain ratios can be misleading, a bi-clonal pattern of immunoglobulin light chain expression or polyphenotypic pattern is rare but does exist, the reported incidence of bi-clonal CLL among all CLL cases varies from 3.4% (1) to 1.4% in a larger study (2). It is known that normal and malignant B-cells could show double productive IGHV rearrangements; however, only one rearrangement will be translated to protein and expressed on the cell surface due to allelic exclusion. Therefore, bi-clonal CLL may reflect lack of allelic exclusion (3). Thus, the absence of two different B-cell receptor rearrangements might be found in bi-clonal CLL.

CASE: A 65-year old man presented without symptoms for a routine check-up at his general practitioner. A CBC leukocytosis of 20.5 x 10^9/L due to an absolute lymphocytosis was observed. No anemia and a discrete thrombocytopenia were present. On peripheral smear the cells were predominantly small, with mature chromatin. Many smudge cells were noted.

PROCEDURE/PROCESS: Detection of phenotypically aberrant and clonal mature lymphocytes is the diagnostic hallmark of CLPD. The EuroFlow group has designed and evaluated an 8-color, 12-marker combination of antibodies aiming at the detection of phenotypically aberrant populations of mature B-, T- and NK-cells in PB, BM, lymph nodes (LN) and other types of body tissues and fluids, which can be used in the diagnostic screening of CLPD (4).

The Euroflow Lymphoid Screening Tube (LST), which includes 12 antibodies conjugated with 8 fluorochromes has the following features: CD45 is selected for the definition of the compartments of mature versus immature lymphocytes, SmCD3 for the identification of T-cells, and both CD19 and CD20 for the selection of B-cells; these two later markers combined with CD45 would allow subsetting of B-cells into mature B-lymphocytes (CD19+, CD20hi and CD45hi) and B-cell precursors (CD19+, CD20-lo/hi, CD45lo). NK-cells should fulfill the criteria for mature lymphocytes (CD45hi, SSClo) in the absence of CD19 and SmCD3 expression and they would typically show reactivity for CD56. Additional markers selected for further subsetting of B, T and NK-cells include (i) SmIgκ and SmIgλ, (ii) CD4, CD8 and CD56, TCRγδ- (CD4+, CD8hi, CD4+/CD8lo, CD4-/CD8lo/TCRγδ- and CD4-/CD8-lo/TCRγδ+ and CD4-/CD8-lo/TCRγδ+) and (iii) CD56, CD38 and CD8, respectively. CD56 is also useful in the positive identification of plasma cells. CD5 provides a more sensitive and/or robust orientation on subsequent analyses, when lymphocyte populations with aberrant or clonal phenotypes are detected (for example, in B-CLPD).
Briefly, CD19+ neoplastic B-cells were selected for each data file with the INFINICYT software (Cytognos SL, Salamanca, Spain), using conventional gating strategies based on CD19 vs. SSC. Due to the use of 2 monoclonal antibodies conjugated with the same fluorochrome, the analysis begins with the identification of the T-cells using CD3 vs. SSC, defining their sub-populations and then excluding them from subsequent analysis of the B cell population.

Special attention must be paid to the co-expression of CD5 (Fig. 1, in pink). Regarding the Kappa and Lambda surface light chain expression, both the CD19+CD5- and the CD19+CD5+ B-cells show expression of kappa and lambda light chains, although at different levels. The pink cells have dimmer expression compared to the normal B-cells in blue (Fig. 2 and 3). Surface light chain immunostaining shows a subset of atypical lymphoid cells positive for kappa (in green), and another subset positive for lambda surface light chain (in orange).

If we look at the co-expression of CD19 and CD20 we can confirm the heterogeneous expression of CD20 as might be expected in this phenotype (Fig 4).

In recent years, a variety of new computational tools have been developed to identify specific cell populations in multidimensional flow cytometry data sets. One such solution is the use of principal component analysis (PCA) of a unified data file with a potentially unlimited number of dimensions (5,6). A PCA-based approach is implemented in Infinicyt software in the automatic population separator (APS) view, where the first (x axis) and second (y axis) principal components are used to produce a bi-dimensional representation of either cell clusters or patient profiles; each principal component is represented by a linear combination of all parameters (light scatter and fluorescence emissions) included in the FCS file(s) under analysis. The APS dot plot features a clear separation of the different B cell clusters based on all the parameters in the LST tube (Fig 5).

<table>
<thead>
<tr>
<th>Pacific Blue</th>
<th>Pacific Orange</th>
<th>FITC</th>
<th>PE</th>
<th>PerCP-Cy5.5</th>
<th>PE-Cy7</th>
<th>APC</th>
<th>APC-H7</th>
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<tbody>
<tr>
<td>CD20 + CD45</td>
<td>CD8 + CD56</td>
<td>Lambda</td>
<td>Kappa</td>
<td>CD19 + TCR γδ</td>
<td>CD3</td>
<td>CD38</td>
<td></td>
</tr>
</tbody>
</table>

Figure 1 Figure 2 Figure 3
The B-CLPD panel is intended to be applied subsequent to the detection of a clonal B-cell population using the LST. In this sample tube 1 of this panel is enough to confirm the suspected diagnosis.

<table>
<thead>
<tr>
<th>Tube</th>
<th>Pacific Blue</th>
<th>Pacific Orange</th>
<th>FITC</th>
<th>PE</th>
<th>PerCP-Cy5.5</th>
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<tbody>
<tr>
<td>1</td>
<td>CD20</td>
<td>CD45</td>
<td>CD23</td>
<td>CD10</td>
<td>CD79b</td>
<td>CD19</td>
<td>CD200</td>
<td>CD43</td>
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</tbody>
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Further characterization of this entity demonstrates positive and homogeneous expression of CD200, CD23 (Fig.6) and CD43, the lambda cluster has positive dimmer expression of CD79b (Fig.7), which contributes to a slight separation between the 2 clones in the APS view (Fig. 8).

EXAMPLE OF REPORT (7)

REPORTABLE RESULTS:
- Polytypic B cells are rare to absent
- 57.2% B cells with the following immunophenotype:
  - sKappa light chains…82.7% (dim)
  - sLambda light chains…17.3% (dim)
- Positive for: CD5, CD20 (heterogeneous), CD23, CD79b (partial dim), CD200 (homogeneous), CD43
- Negative for: CD10, CD38
COMMENT: Immunophenotype favors bi-clonal chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL).

FINAL NOTE:

The presence of two monoclonal populations raises the question of the diagnostic criteria for MBL and CLL. When considered separately, the CD19+ lambda population can be regarded as MBL, as this population at time of subtyping consisted of less than 5 x 10^9/L clonal lymphocytes (2.0 x 10^9/L).

It is unclear whether all clonal cells need to be part of one single clonal population to fulfill the WHO criteria, nevertheless the CD19+ kappa population can be regarded as CLL on its own, as this population at time of subtyping consisted of more than 5 x 10^9/L clonal lymphocytes (11.3 x 10^9/L). In contrast to the WHO criteria, the International Workshop on CLL formulation defines 5 x 10^9/L total B-lymphocytes, not only the clonal B-cells, as cutoff for diagnosing CLL, so our case would be classified as CLL according to both criteria (8,9).

No difference could be made morphologically between both clones.

The only genetic feature identified was 13q- by FISH. In the literature, the most frequent cytogenetic abnormality in bi-clonal CLL found was del 13q (62.2%), followed by trisomy 12 (15.9%). (2)

References:

Reviewed and approved by: Mike Linden and Mike Keeney

For any questions on this module or any other suggestions, please email info@cytometry.org

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