Flow Cytometric Analysis of Surface Light Chain Expression Patterns in B-Cell Lymphomas Using Monoclonal and Polyclonal Antibodies

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Abstract

Light chain (LC) expression by flow cytometry (FC) in B cell non-Hodgkin lymphomas (B-NHLs) can occasionally be detected with one anti-LC antibody but not with another. We retrospectively analyzed 564 four-color FC files from B-NHLs, assessing LC staining with monoclonal antibodies (mAbs) and polyclonal antibodies (pAbs). Discrepancies in LC expression between mAbs and pAbs were present in 9.2% of cases, mainly in chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL; 11.1%), diffuse large B-cell lymphoma (DLBCL; 10.2%), follicular lymphoma (9.5%), and mantle cell lymphoma (11.1%) and most frequently in body fluids. Equal proportions of cases were LC+ only with pAbs (4.8%) or mAbs (4.4%). Negative LC expression with both antibodies was present in 7.5% of cases, most frequently in DLBCL (21.6%) and body fluids (27.6%). Evaluation with both mAbs and pAbs increases the sensitivity for LC detection, with no single reagent outperforming the other, although CLL/SLL preferentially showed LC expression with pAbs.

Immunoglobulin light chain (LC) expression analysis has a critical role in the flow cytometric evaluation of B-cell non-Hodgkin lymphomas (B-NHLs). The presence of a distinct B-cell population expressing only 1 type of immunoglobulin LC (κ or λ) essentially establishes a clonal B-cell process and supports a diagnosis of lymphoma. However, flow cytometry does not always detect surface LC expression on lymphoma cells; a phenomenon most commonly reported in diffuse large B-cell lymphoma but also described in other B-NHLs. Although this apparent lack of surface immunoglobulin is postulated to be due to posttranscriptional defects of the immunoglobulin molecule, few published studies have suggested that the choice of anti-LC antibody is one of the main factors determining whether expression of surface LC can be demonstrated. Currently, monoclonal antibodies (mAbs) and polyclonal antibodies (pAbs) are commercially available for the clinical assessment of LC expression by flow cytometry, but neither has been consistently shown to be superior to the other.

While routinely using monoclonal and polyclonal anti-LC antibodies in flow cytometric evaluations of lymphoma, we have observed B-NHLs that show LC restriction with one set of antibodies but appear to lack LC expression with the other. This discrepant pattern of surface LC expression using mAbs and pAbs has been previously documented in small series but the significance of this finding has not been systematically studied in a large series of diverse B-NHL types and specimens.

Materials and Methods

We retrospectively reviewed the flow cytometry laboratory database at Dynacare Laboratories/Medical College of Wisconsin, Milwaukee, for all specimens with a diagnosis of B-NHL, including diagnostic and follow-up specimens, received between 2006 and 2010. Only cases that were
Aliquots of 5 × 10^5 cells were incubated with antibodies conjugated in RPMI 1640/penicillin-streptomycin culture medium, followed by 3 rounds of centrifugation. All specimens were resuspended in RPMI 1640/penicillin-streptomycin culture medium. Aliquots of 5 × 10^3 cells were incubated with antibodies conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein (PerCP), and allophycocyanin (APC) at 2°C to 8°C in the dark for 20 minutes. Cells were then washed with phosphate-buffered saline, fixed with 0.5% paraformaldehyde, and analyzed within 24 hours of staining.

Between January 2006 and February 2009, at least 30,000 events were acquired routinely using a FACSCalibur flow cytometer with CellQuest software (Becton Dickinson, San Jose, CA); between March 2009 and December 2010, at least 75,000 events were acquired routinely using a FACSCanto flow cytometer with FACSDiva software (Becton Dickinson, San Jose, CA). Two different surface LC tubes were included for each case, one using FITC-conjugated anti-κ (TB28-2) with PE-conjugated anti-λ (I-155-2) mAbs (Becton Dickinson, San Jose) and another using PE-conjugated anti-κ (goat) with FITC-conjugated anti-λ (goat) pAbs (Coulter-Immunotech, Brea, CA). Each of the LC tubes also included APC- and PerCP-conjugated antibodies directed against 2 of the following antigens (variable, depending on the panel): CD5, CD19, CD20, CD22, and/or CD38 (Becton Dickinson, Franklin Lakes, NJ). Isotype controls were performed in all cases. Intracytoplasmic LC assessment was not performed.

Data Analysis
Data were analyzed using cluster analysis and Paint-A-Gate software (Becton Dickinson, San Jose). Flow cytometry acquisition files were reanalyzed retrospectively by one of the investigators (P.H.). Lymphoma cells were identified as cohesive clusters of events showing aberrant surface antigen expression and/or light scatter characteristics relative to normal B-cell patterns. Positivity for LC expression in each of the LC tubes was determined by deviation from the diagonal in a k vs λ plot, with internal T cells serving as negative controls, as previously described.4 Nonneoplastic B cells, when present, were identified based on their normal expression of surface antigens and served as internal positive controls for κ and λ staining.

Classification of Cases
Flow cytometric results were classified according to the presence or absence of LC staining in the monoclonal and polyclonal LC tubes. A discrepant LC staining pattern was defined as no detectable LC expression with one set of LC reagents and positive LC expression with the other. Cases with equivocal LC expression were reviewed by 2 investigators (P.H. and A.M.H.) and classified by consensus.

The B-NHL diagnoses were made according to the World Health Organization 2001 and 2008 criteria,9,10 using a combination of morphologic, cytogenetic, molecular, clinical, and radiologic findings. Wright-Giemsa-stained cytocentrifuged preparations were available for review in all cases; H&E-stained histologic and/or Wright-Giemsa-stained cytologic studies were available in most cases. In the absence of sufficient information for final classification, small to medium-sized lymphoid proliferations were classified according to CD5 and CD10 expression by flow cytometry, and medium- to large-sized lesions with aggressive histologic features were classified as diffuse large B-cell lymphoma/Burkitt (DLBCL/BL).

Statistical Analysis
Categorical variables were analyzed by using the Fisher exact test (2-tailed) using GraphPad Prism software, version 5.0b (GraphPad Software, San Diego, CA). A P value less than .05 was considered statistically significant.

Results
We analyzed 564 specimens from 436 patients. Table I summarizes the B-NHL diagnoses and specimen types for each case, one using FITC-conjugated anti-κ (TB28-2) with PE-conjugated anti-λ (I-155-2) mAbs (Becton Dickinson, San Jose) and another using PE-conjugated anti-κ (goat) with FITC-conjugated anti-λ (goat) pAbs (Coulter-Immunotech, Brea, CA). Each of the LC tubes also included APC- and PerCP-conjugated antibodies directed against 2 of the following antigens (variable, depending on the panel): CD5, CD19, CD20, CD22, and/or CD38 (Becton Dickinson, Franklin Lakes, NJ). Isotype controls were performed in all cases. Intracytoplasmic LC assessment was not performed.

<table>
<thead>
<tr>
<th>Specimen</th>
<th>No. (%): 564 cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone marrow</td>
<td>168 (29.8)</td>
</tr>
<tr>
<td>Periphera blood</td>
<td>151 (26.8)</td>
</tr>
<tr>
<td>Lymph node</td>
<td>117 (20.7)</td>
</tr>
<tr>
<td>Soft tissue mass</td>
<td>60 (10.6)</td>
</tr>
<tr>
<td>Other tissues</td>
<td>33 (5.9)</td>
</tr>
<tr>
<td>Body fluid</td>
<td>29 (5.1)</td>
</tr>
<tr>
<td>Bone lesion</td>
<td>6 (1.1)</td>
</tr>
</tbody>
</table>

Table I
Diagnoses and Specimen Types for 564 Cases

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>No. (%): 564 cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chronic lymphocytic leukemia/small lymphocytic lymphoma</td>
<td>235 (41.7)</td>
</tr>
<tr>
<td>Diffuse large B-cell lymphoma</td>
<td>88 (15.6)</td>
</tr>
<tr>
<td>Follicular lymphoma</td>
<td>74 (13.1)</td>
</tr>
<tr>
<td>Mantle cell lymphoma</td>
<td>27 (4.8)</td>
</tr>
<tr>
<td>Marginal zone lymphoma</td>
<td>24 (4.3)</td>
</tr>
<tr>
<td>Lymphoplasmacytic lymphoma</td>
<td>18 (3.2)</td>
</tr>
<tr>
<td>Diffuse large B-cell lymphoma/Burkitt lymphoma</td>
<td>9 (1.6)</td>
</tr>
<tr>
<td>Hairy cell leukemia</td>
<td>6 (1.1)</td>
</tr>
<tr>
<td>Burkitt lymphoma</td>
<td>4 (0.7)</td>
</tr>
<tr>
<td>CD5+CD10−</td>
<td>49 (8.7)</td>
</tr>
<tr>
<td>CD5+</td>
<td>21 (3.7)</td>
</tr>
<tr>
<td>CD10+</td>
<td>9 (1.6)</td>
</tr>
</tbody>
</table>

Data were analyzed using cluster analysis and Paint-A-Gate software (Becton Dickinson, San Jose). Flow cytometry acquisition files were reanalyzed retrospectively by one of the investigators (P.H.). Lymphoma cells were identified as cohesive clusters of events showing aberrant surface antigen expression and/or light scatter characteristics relative to normal B-cell patterns. Positivity for LC expression in each of the LC tubes was determined by deviation from the diagonal in a k vs λ plot, with internal T cells serving as negative controls, as previously described.4 Nonneoplastic B cells, when present, were identified based on their normal expression of surface antigens and served as internal positive controls for κ and λ staining.
cases included in the study. Based on the pathology material and clinical information available for review, 79 cases could not be classified into specific World Health Organization 2001 and 2008 diagnostic categories and were classified based on expression of CD5 and CD10. The most common types of specimens analyzed included bone marrow (29.8%), peripheral blood (26.8%), and lymph nodes (20.7%). Body fluids accounted for 5.1% of specimens and consisted of 18 pleural, 5 peritoneal, 4 cerebrospinal, 1 bronchial, and 1 vitreous fluid.

Positivity for LC was observed with mAbs and pAbs in 470 cases (83.3%). In 52 cases (9.2%), a discrepant LC expression pattern was shown. Of these discrepant cases, 25 (4.4%) showed LC expression only with mAbs, while 27 (4.8%) were positive for LC expression only with pAbs. In 42 cases (7.4%), both antibody sets were LC negative. Representative cases for each of these LC expression patterns are illustrated in Image 1.

Of the 485 cases with specific lymphoma diagnoses, 46 (9.5%) showed a discrepant LC expression pattern, including 26 (11.1%) of 235 chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL) cases, 3 (11%) of 27 mantle cell lymphomas (MCLs), 9 (10%) of 88 DLBCLs, 7 (9%) of 74 follicular lymphomas (FLs), and 1 (6%) of 18 lymphoplasmacytic lymphomas. Of these discrepant cases with specific lymphoma diagnoses, the pAb+/mAb– pattern was most commonly observed in CLL/SLLs (18/26 [69%]) compared with other B-NHLs (7/20 [35%]; P = .036). No

**Image 1** Representative dot plots from non-Hodgkin B-cell lymphoma cases showing tumor cells (red) with different light chain expression patterns. Blue, normal B cells; green, T cells. A, Monoclonal antibody (mAb)+/polyclonal antibody (pAb)– pattern in a case of diffuse large B-cell lymphoma (DLBCL) involving the bone marrow. B, mAb–/pAb+ pattern in a chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL) involving the bone marrow. C, Biclonal CLL/SLL in the peripheral blood, with one clone (yellow) showing an mAb+/pAb– pattern and another clone (red) showing light chain restriction with both antibody sets. D, mAb–/pAb– pattern in a case of DLBCL from a lymph node biopsy.
other statistically significant relationships between pattern and diagnosis were identified. Discrepancies in LC expression were not present in hairy cell leukemia, marginal zone lymphoma (MZL), BL, or DLBCL/BL.

A discrepant LC expression pattern was most often observed in body fluids (6/29 [21%]) compared with 18 (10.7%) of 168 bone marrow samples ($P = .13$), 13 (8.6%) of 151 peripheral blood samples ($P = .05$), 4 (7%) of 60 soft tissue samples, 7 (6.0%) of 117 lymph nodes ($P = .013$), and 4 (13%) of 30 other tissue types. The discrepant body fluid cases consisted of 2 CLL/SLLs, 1 DLBCL, 1 MCL, and 2 unclassifiable cases in 5 pleural fluid specimens and 1 peritoneal fluid specimen.

Negative LC expression with both antibody sets was observed in 42 (7.4%) of 564 cases and was most commonly observed in cases of DLBCL (19/88 [22%]) compared with 5 (7%) of 74 FLs ($P = .008$), 13 (5.5%) of 235 CLL/SLLs ($P < .001$), 1 (4%) of 24 MZLs ($P = .07$), and 1 (4%) of 27 MCLs ($P = .04$). In addition, negative LC expression was more common in body fluids (8/29 [28%]), including 5 pleural fluid specimens, 2 peritoneal fluid specimens, and 1 vitreous fluid specimen, than in other specimen types (34/535 [6.4%]; $P < .001$). Normal residual polytypic B cells were detectable in 20 (48%) of the 42 cases that were negative for LC with both reagents (Image 1D).

Discussion

The demonstration of LC restriction in B cells (with the implication of monoclonality) is the traditional foundation of B-cell lymphoma diagnosis by flow cytometry. However, this approach has occasional pitfalls and there is increasing recognition that neoplastic B-cell populations are often identifiable based on abnormal patterns of surface antigen expression (exclusive of LC restriction). In this retrospective study, we found that discrepant LC expression patterns, defined as the detection of LC expression with one antibody set and not another, occur relatively frequently (9.2% of B-NHLs in our large series). Moreover, we found that the performance of each antibody set is very similar among B-NHLs (4.4% of cases positive only with mAbs vs 4.8% positive only with pAbs), underscoring the importance of using both reagents to increase the sensitivity of LC detection by flow cytometry in B-NHLs. These results represent novel findings, as our study is the first to comprehensively examine a large, diverse cohort of B-cell lymphomas in various specimen types using monoclonal and polyclonal LC antibodies with 4-color flow cytometry.

In our series, all B-NHLs with discrepant LC expression patterns were seen almost entirely in 4 diagnostic categories, CLL/SLL, MCL, DLBCL, and FL, at a fairly equal frequency (approximately 10% of each diagnostic category). LC discrepancies were not observed with MZLs, BLs, or hairy cell leukemia.

![Figure 1](image1.png)

**Figure 1** Frequency of discrepant light chain staining patterns (black and gray) and negative staining pattern (white) among different non-Hodgkin B-cell lymphoma diagnoses. BCL, Burkitt lymphoma; CLL/SLL, chronic lymphocytic leukemia/small lymphocytic lymphoma; DLBCL, diffuse large B-cell lymphoma; FL, follicular lymphoma; HCL, hairy cell leukemia; LPL, lymphoplasmacytic lymphoma; mAb, monoclonal antibody; MCL, mantle cell lymphoma; MZL, marginal zone lymphoma; pAb, polyclonal antibody.

![Figure 2](image2.png)

**Figure 2** Frequency of discrepant light chain staining patterns (black and gray) and negative staining pattern (white) among different types of specimens. LN, lymph node; mAb, monoclonal antibody; pAb, polyclonal antibody.
leukemias and were found in only 1 case of lymphoplasmacytic lymphoma, although fewer cases of these lymphoma types were included in our cohort. As mentioned, across all the lymphomas, the discrepant patterns of mAb−/pAb+ and mAb+/pAb− were observed with equal frequency. However, the discrepant pattern of mAb−/pAb+ was most commonly observed in CLL/SLL compared with other B-NHLs. As the majority of CLL/SLLs with this discrepancy demonstrated only LC expression by pAbs and this neoplasm characteristically has dim LC expression, our data suggest that dim LC expression is best detected with pAbs in this lymphoma type.

Possible explanations for a discrepant LC expression pattern (and a negative LC pattern) include loss of the recognized epitope(s) for one of the antibodies due to immunoglobulin gene mutations and defective transcription or posttranslational modifications of the LC molecule including the membrane-anchoring portion.5,6 Studies of LC− lymphomas have demonstrated B-cell receptor gene rearrangements to be as frequent as in their LC+ counterparts,6 supporting transcriptional and posttranslational defects as a mechanism for discrepant or negative LC patterns. Alternatively, it could be proposed that low levels of expression of surface immunoglobulin, in combination with potential differences in the sensitivity of the antibody sets, could also have resulted in undetectable LC expression with one of the antibodies and not the other.3 However, the nearly equal percentage of cases positive with mAbs only and with pAbs only in our cohort argues against this hypothesis.

Negativity for LC expression with both antibody sets was observed in 7.4% of cases in our cohort, which is consistent with the published range in the flow cytometry literature of 2.25% to 12.2%.1-3,14 However, as our definition of LC negativity was most similar to that used by Kaleem et al3 and Li et al4 comparisons between our data (7.4%) and the data of these 2 studies, 3.4% and 2.25% respectively, is most relevant. The explanation for the higher frequency of LC negativity observed in our study is not entirely clear, especially given that Li et al4 used both mAbs and pAbs in their analyses. One possibility is that the differences were due to a different distribution of lymphoma types; however, neither report describes the numbers of specific lymphoma types in their cohorts. Another possible explanation is differences in specimen types analyzed: Kaleem et al3 analyzed only lymph nodes, in contrast with our analysis, which included body fluids (found to have high rates of LC negativity; see subsequent text). LC negativity in our study was not likely due to technical failure because in almost half of the LC− cases, polyclonal B cells were present in the background, serving as internal positive controls.

LC negativity was observed most frequently in DLBCL, with DLBCL cases accounting for almost half of the LC− cases in our cohort. Indeed, lack of surface immunoglobulin in B-NHLs has been most frequently described in DLBCL,1,2,3,4 with variable frequency depending on the technique used for immunoglobulin detection,5,6 although this finding has not been comprehensively studied to date. Approximately one fifth of the DLBCLs showed LC negativity by our assessment, which is in agreement with the findings by Tomita et al5 in which 24% of their DLBCL cohort showed lack of LC expression. Lack of LC expression was also observed in FLs and CLL/SLLs and rarely in MCL and MZL in our study, similar to previous studies.3,4

Several authors have considered a lack of surface LC expression in mature B-cell lymphomas to represent an aberrant immunophenotypic feature and, thus, a marker of neoplasia.3,4 However, others have disputed this assertion.7 Notably, normal germinal center cells down-regulate surface immunoglobulin. However, this down-regulation is incomplete, and germinal center cell populations defined by CD10 expression or bright coexpression of CD20 and CD38 have demonstrable partial polyclonal LC expression,16 in contrast with the complete lack of LC expression seen in the LC− cases in our cohort. The 3 reactive lymph nodes with expanded, CD10dim+, LC− B-cell populations reported by Zhao et al7 may have represented examples of this phenomenon. Lymphoma diagnoses in the present study were made based on a combination of immunophenotypic aberrancies, morphologic features, and cytogenetic abnormalities; the lack of LC expression (while arguably an aberrant immunophenotypic finding) was not used as the sole criterion for a neoplasia diagnosis.

We found an overrepresentation of a negative LC expression pattern and a discrepant LC expression pattern in body fluids. Several authors have previously reported lack of LC expression by flow cytometry (presumably using single antibody reagents) in these specimen types.15,18 In our practice, we periodically encounter LC− reactive B cells in serous effusions (with both antibody sets), particularly from the pleural cavity (unpublished data). In some cases, polyclonal LC expression on the B cells can be elicited with prolonged 37°C incubation and/or increasing antibody reagent concentrations. The mechanism of this technical artifact is unclear, but it seems to be limited to this specimen type. Therefore, lack of LC expression should be interpreted with caution in body cavity effusion samples.

We describe a discrepant pattern of LC expression by 4-color flow cytometry in a small but significant percentage of CLL/SLLs, MCLs, DLBCLs, and FLs. The concurrent use of anti-LC mAbs and pAbs in the routine flow cytometric evaluation of B-NHLs increases the sensitivity for demonstrating LC restriction, with neither reagent set showing a clear sensitivity advantage in general, although CLL/SLLs seem to preferentially show LC expression with pAbs. Instead of concurrent use of both surface antibody sets, a laboratory
may consider use of one set as a screen, with the addition of the other set if LC expression is not identified initially. Even with 2 sets of LC reagents, however, surface LC negativity is encountered in a minor subset of lymphomas (approximately 8%, predominantly DLBCLs), consistent with previous literature data. Cytoplasmic LC staining may increase the sensitivity of LC detection in B-NHLs, although there are limited data in the literature to support this approach.19

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References