CD200 Flow Cytometric Assessment and Semiquantitative Immunohistochemical Staining Distinguishes Hairy Cell Leukemia From Hairy Cell Leukemia-Variant and Other B-Cell Lymphoproliferative Disorders

Vinodh Pillai, MD, PhD, Olga Pozdnyakova, MD, PhD, Karry Charest, Betty Li, MS, Aliakbar Shahsafaei, MS, and David M. Dorfman, MD, PhD

From the Department of Pathology, Brigham and Women’s Hospital and Harvard Medical School, Boston, MA.

Key Words: Chronic lymphocytic leukemia; Mantle cell lymphoma; Hairy cell leukemia; Hairy cell leukemia-variant

ABSTRACT

Objectives: To evaluate CD200 expression in B-cell proliferative disorders.

Methods: We analyzed 180 recent specimens of B-cell neoplasms for CD200 expression by flow cytometric immunophenotypic analysis, which is better able to assess relative intensity of staining than immunohistochemical staining.

Results: We found that hairy cell leukemia exhibits a high level of staining for CD200 in comparison to other B-cell lymphoproliferative disorders, including hairy cell leukemia-variant (HCL-V), marginal zone lymphoma, and lymphoplasmacytic lymphoma. We confirmed this observation by semiquantitative immunohistochemical staining.

Conclusions: Assessment of the CD200 expression level is helpful to distinguish HCL from HCL-V and other B-cell lymphoproliferative disorders and in the differential diagnosis of B-cell neoplasms in general.

CD200 (OX-2 antigen), a type I immunoglobulin superfamily membrane glycoprotein, is widely expressed in multiple cell types, including B cells, a subset of T cells, dendritic cells, endothelial cells, and in the peripheral and central nervous system. It interacts with CD200R, an immunoglobulin superfamily inhibitory receptor expressed primarily on myeloid/monocyte lineage cells, and has a suppressive effect on T-cell–mediated immune response. CD200 has been reported to be expressed by plasma cells in multiple myeloma by flow cytometric immunophenotyping, in acute myeloid leukemias by gene expression array analysis, and in a number of carcinomas and other malignant neoplasms, including malignant melanoma, by gene expression array analysis. CD200 is a marker of poor prognosis in multiple myeloma and acute myeloid leukemia and has been associated with tumor progression in a number of carcinomas, probably through its immunosuppressive effect on the host immune system.

Upon completion of this activity you will be able to:

• describe the expression of CD200 in different hematologic malignancies.
• discuss the utility of flow cytometric assessment of CD200 expression in the diagnosis of hairy cell leukemia.

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CD200 expression has been upregulated in chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL) when compared with normal B cells by flow cytometric immunophenotyping. CD200 expression by neoplastic cells downregulates the Th1 immune response and suppresses the antitumor immune response in an animal model of CLL.

We previously studied the expression of CD200 in a wide range of B-cell lymphoproliferative disorders and other B-cell–derived neoplasms, including all of the most common B-cell neoplasms encountered in clinical practice, by immunohistochemical staining to determine its utility as an immunophenotypic marker in the diagnosis and analysis of these diseases. We and others found that, in addition to CLL/SLL, CD200 is expressed in a number of neoplasms derived from B cells, including hairy cell leukemia (HCL) and B-lymphoblastic leukemia/lymphoma (B-ALL). Neoplastic CD200 is expressed by the neoplastic B cells in mediastinal large B-cell lymphoma and classic Hodgkin lymphoma but not in diffuse large B-cell lymphoma (DLBCL). Neoplastic cells in nodular lymphocyte-predominant Hodgkin lymphoma are CD200 negative, but nonneoplastic T cells that rosette the neoplastic lymphocyte-predominant cells are consistently CD200 positive. We have shown previously that these T cells express immunophenotypic markers of follicular helper T cells, including PD-1 and CXCL13. In a subsequent study, we found that CD200 is expressed by follicular helper T cells in reactive lymphoid tissue and by the neoplastic cells in angioimmunoblastic T-cell lymphoma. In contrast, only some cases of T-cell neoplasms other than angioimmunoblastic T-cell lymphoma were immunoreactive for CD200.

Here we report on flow cytometric immunophenotypic analysis of a large number of samples of B-cell neoplasms encountered in clinical practice to determine whether assessment of the relative intensity of CD200 staining is helpful for differential diagnosis.

Materials and Methods

Patients

This study was approved by the Brigham and Women’s Hospital institutional review board.

Between October 2010 and January 2012, the Brigham and Women’s Hospital Hematology Laboratory performed flow cytometric analysis for CD200 expression on 180 specimens (bone marrow aspirates, lymph nodes, peripheral blood, body fluids, and other tissues) involved by a B-cell lymphoproliferative disorder. Final pathologic diagnoses were established according to the criteria of the 2008 World Health Organization classification using morphology, immunohistochemical staining, and cytogenetic analysis where applicable. There were 10 cases of HCL studied by flow cytometric immunophenotypic analysis from patients ranging in age from 33 to 78 years (mean, 56 years), including 8 men and 2 women. Patients presented with cytopenias (8/10) and/or splenomegaly (4/10) and had bone marrow involvement (9/10) by a clonal population of intermediate-sized lymphocytes with cytoplasmic projections. Neoplastic B lymphocytes coexpressed CD11c, CD25, and CD103 in all 10 cases. There were 4 cases of hairy cell leukemia-variant (HCL-V) studied by flow cytometric immunophenotypic analysis from patients ranging in age from 62 to 98 years (mean, 74 years), including 2 men and 2 women. Patients presented with lymphocytosis (3/4) and/or splenomegaly (2/4) and had peripheral blood and/or bone marrow involvement (3/4) by a clonal population of lymphocytes with villous projections. Neoplastic B lymphocytes coexpressed CD11c and CD103 and were negative for CD25 in all 4 cases.

Flow Cytometric Analysis

Six-color immunophenotypic analysis was performed using a FACSCanto II (BD Biosciences, San Jose, CA) flow cytometer with simultaneous assessment of CD19, CD20, CD5, CD10, CD23, and CD200. The flow cytometric panel used was CD5 FITC, CD200 PE, CD19 PerCP-Cy5.5, CD10 PE-Cy7, CD23 APC, and CD20 APC-H7 (all from BD Biosciences). Flow cytometric data were analyzed with FACS DIVA software (BD Biosciences). Lymphocytes were first gated using the forward vs side scatter. Relevant neoplastic populations were then specifically gated based on their CD19, CD20, CD5, or CD10 expression. Mean fluorescence intensity (MFI) of the neoplastic population in the CD200 PE channel was determined using the statistics options available in FACS DIVA.

Immunohistochemistry

Immunohistochemistry was performed on 5-μm-thick formalin-fixed, paraffin-embedded tissue sections using a standard indirect avidin-biotin horseradish peroxidase method and 3,3’-diaminobenzidine color development, as previously described. Briefly, slides were soaked in xylene, passed through graded alcohols, and put in distilled water. The sections were blocked for peroxidase activity with 3% hydrogen peroxide in ethanol for 15 minutes and washed under the running water for 5 minutes. Slides were pretreated with 10 mmol/L citrate, pH 6.0 (for CD200), or 1 mmol/L EDTA buffer, pH 8.0 (for T-bet), in a pressure cooker (Pacific Southwest Lab Equipment, Vista, CA), followed by washing in distilled water. All further steps were performed at room temperature in a hydrated chamber. Immunostaining with goat anti–human CD200 polyclonal antibody (R&D Systems, Minneapolis, MN) or T-bet (monoclonal antibody 4B10) was compared with that of goat immunoglobulin G (IgG) control antibody or mouse IgG control antibody diluted to identical protein concentration to confirm staining specificity.
Statistical Analysis
Statistical analysis was performed using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA).

Results
CD200 Expression by Flow Cytometric Immunophenotypic Analysis
A total of 180 specimens of bone marrow, lymph nodes, peripheral blood, body fluids, and other tissues known to be involved by a B-cell lymphoproliferative disorder were analyzed for CD200 expression, along with CD19, CD20, CD5, CD10, and CD23, by 6-color flow cytometric analysis. Findings were correlated with morphologic, immunophenotypic, and cytogenetic findings to verify the final diagnosis. Results are summarized in Table 1, Table 2, and Figure 1.

We found that HCL cases exhibited very high levels of CD200 staining, with an MFI of 15,411, when compared with HCL-V (CD200 MFI = 742), marginal zone lymphoma (MZL; CD200 MFI = 913), and lymphoplasmacytic lymphoma (LPL; CD200 MFI = 2,131) (Figure 1). The differences in CD200 MFI results for HCL and these other B-cell lymphoproliferative disorders were statistically significant (Tables 1 and 2). On the basis of these results, along with the observation that CD5-positive T cells exhibit CD200 staining with a mean ± SD MFI of 249 ± 146 (MFIs ranging from 140-643 in 20 patient samples), as well as prior immunohistochemical staining studies indicating that cases of HCL are routinely positive for CD200 and cases of MZL are routinely negative for CD200, we considered an MFI of more than 1,000 to represent positive staining for CD200 and an MFI of 1,000 or less to represent negative staining for CD200. We examined CD200 staining in reactive B cells in 12 peripheral blood samples from patients evaluated for the presence of a B-cell lymphoproliferative disorder and found that the MFI of CD200 staining of B cells ranged from 1,516 to 2,921 (mean ± SD, 2,300 ± 469).

Table 1
CD200 Expression in B-Cell Lymphoproliferative Disorders (B-LPDs) by Flow Cytometric Analysisa

<table>
<thead>
<tr>
<th>B-LPD</th>
<th>CD200 MFI, Mean ± SD (Range)</th>
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<tr>
<td>CLL/SLL</td>
<td>5,965 ± 3,123 (1,930-14,126)</td>
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<tr>
<td>MCL</td>
<td>379 ± 315 (80-1,143)</td>
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<tr>
<td>FL</td>
<td>521 ± 286 (15-1,195)</td>
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<tr>
<td>MZL</td>
<td>913 ± 680 (304-2,786)</td>
</tr>
<tr>
<td>LPL</td>
<td>2,131 ± 1,996 (169-8,000)</td>
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<tr>
<td>HCL</td>
<td>15,411 ± 6,156 (7,614-23,252)</td>
</tr>
<tr>
<td>HCL-V</td>
<td>742 ± 651 (137-1,513)</td>
</tr>
<tr>
<td>DLBCL</td>
<td>1,279 ± 1,887 (3-5,931)</td>
</tr>
<tr>
<td>B-ALL</td>
<td>6,760 ± 4,646 (2,425-13,605)</td>
</tr>
<tr>
<td>BL</td>
<td>522 ± 411 (160-1,064)</td>
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</tbody>
</table>

B-ALL, B-lymphoblastic leukemia; BL, Burkitt lymphoma; CLL/SLL, chronic lymphocytic leukemia/small lymphocytic lymphoma; DLBCL, diffuse large B-cell lymphoma; FL, follicular lymphoma; HCL, hairy cell leukemia; HCL-V, hairy cell leukemia-variant; LPL, lymphoplasmacytic lymphoma; MCL, mantle cell lymphoma; MFI, mean fluorescence intensity; MZL, marginal zone lymphoma.

* P < .001 for CLL/SLL vs MCL, CLL/SLL vs FL, HCL vs HCL-V, HCL vs MZL, and HCL vs LPL. P = .03 for B-ALL vs BL.

Table 2
CD200 Expression in B-Cell Lymphoproliferative Disorders (B-LPDs) by Immunohistochemical Analysisa

<table>
<thead>
<tr>
<th>B-LPD</th>
<th>CD200+</th>
<th>CD200–</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCL</td>
<td>23</td>
<td>0</td>
</tr>
<tr>
<td>HCL-V</td>
<td>1</td>
<td>9</td>
</tr>
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</table>

HCL, hairy cell leukemia; HCL-V, hairy cell leukemia-variant.

* P < .001 for HCL vs HCL-V.
We found that cases of CLL/SLL exhibited CD200 staining with an MFI of 5,965, in contrast to cases of mantle cell lymphoma (MCL) and follicular lymphoma (FL), which had MFI for CD200 staining of 379 and 521, respectively. The difference in CD200 staining for CLL/SLL vs MCL and CLL/SLL vs FL was statistically significant (P < .001; Table 1) and correlated with prior immunohistochemical staining studies. Similarly, cases of B-ALL exhibited a high CD200 MFI (6,760) compared with cases of Burkitt lymphoma (BL; CD200 MFI = 522), which was statistically significant (P < .001; Fisher exact probability test; Table 1). A final group of cases studied consisted of 2 cases of B-prolymphocytic leukemia with a CD200 MFI of 1,279, and clonal populations of B cells lacking additional clinical and/or morphologic data to support a specific diagnosis, with a range of CD200 MFI values (data not shown).

**CD200 Expression by Immunohistochemical Staining and Semiquantitative Immunohistochemical Staining**

Because of the relatively small number of cases of HCL and HCL-V studied by flow cytometric immunophenotypic analysis, we performed immunohistochemical staining for CD200 on additional cases of HCL and HCL-V to verify the above flow cytometric findings. T-bet, a T-cell–associated transcription factor widely expressed in HCL, MZL, and CLL/SLL, served as a positive control. Most HCL and HCL-V cases were positive for T-bet (21/23 and 9/10, respectively). In contrast, all cases of HCL were positive for CD200 (23/23), and only 1 case of HCL-V (1/10) was positive for CD200 (P < .001; Fisher exact probability test; Table 2). When these results were combined with the results from flow cytometric analysis, 33 of 33 cases of HCL were positive for CD200, and 2 of 12 cases of HCL-V were positive for CD200 (P < .001; Fisher exact probability test).

We performed semiquantitative immunohistochemical staining for CD200 to further evaluate the differences in staining intensity observed for different B-cell lymphoproliferative disorders by flow cytometric immunophenotypic analysis. Image 4 shows CD200 staining of CLL/SLL, B-ALL, HCL, and HCL-V under normal staining conditions (1:250 dilution) and also with 4-fold dilution of antibody (1:1,000 dilution). CLL/SLL and B-ALL exhibited low to moderate intensity staining for CD200 in contrast to HCL, which showed higher intensity staining. HCL-V was negative for CD200. As the concentration of CD200 antibody decreased, staining intensity in CLL/SLL, B-ALL, and HCL

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**Image 1** CD200 expression in hairy cell leukemia (HCL) (A) and hairy cell leukemia-variant (HCL-V) (B). A case of HCL is CD19 and CD20 positive with coexpression of CD11c, CD25, and CD103 (not shown), and CD200 (mean fluorescent intensity [MFI] = 17,964; population shown in green) and is negative for CD5 and CD23 (not shown). B, A case of HCL-V is CD19 and CD20 positive with coexpression of CD11c and CD103 (not shown), has weak coexpression of CD200 (MFI = 1,043; population shown in green), and is negative for CD5, CD23, and CD25 (not shown).
decreased, but the higher relative intensity of HCL staining could be seen. When the antibody was further diluted to 1:2,500, CD200 staining of CLL/SLL and B-ALL greatly decreased in comparison to HCL, which continued to stain for CD200, although at a low level, in comparison to CLL/SLL and B-ALL, corresponding to the relative staining intensities of these neoplasms by flow cytometric immunophenotypic analysis (Table 1 and Figure 1).
Here we report on CD200 expression in B-cell lymphoproliferative disorders as assessed by flow cytometric immunophenotypic analysis. In contrast to immunohistochemical staining, flow cytometric analysis provides a quantitative assessment of staining intensity, allowing a comparison of CD200 expression in various B-cell neoplasms. We find that HCL exhibits the highest level of staining for CD200 when compared with other B-cell lymphoproliferative disorders, including HCL-V, MZL, and LPL, and confirm this observation by semiquantitative immunohistochemical staining. It should be noted, however, that the patterns of CD200 staining and not absolute CD200 MFI are most useful for differential diagnosis because of possible variation in MFI that may be encountered with different instrumentation, antibodies, and fluorochromes employed for analysis. The findings reported should be helpful in the diagnostic evaluation of HCL and other B-cell lymphoproliferative disorders because these neoplasms may have some overlapping clinical and immunophenotypic findings but are treated differently. HCL is effectively treated with purine analogues, such as cladribine and pentostatin, while HCL-V does not typically respond to these chemotherapeutic agents but has been successfully treated with rituximab and anti-CD22 immunotoxin. MZL also responds to treatment with rituximab, as does LPL. A number of B-cell lymphoproliferative disorders are thought to be derived from B lymphocytes in a state of post-germinal center differentiation/maturation, including HCL, HCL-V, MZL, and LPL. For example, HCL is thought to be derived from a late activated memory B cell.

**Image 4** Semiquantitative staining for CD200 in chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL), B-lymphoblastic leukemia (B-ALL), hairy cell leukemia (HCL), and hairy cell leukemia-variant (HCL-V). Staining was performed at 1:250 antibody dilution, demonstrating typical relative staining intensities of the 4 neoplasms, with greatest staining seen in HCL and absence of staining in HCL-V. The relatively greater intensity of CD200 staining in HCL compared with CLL/SLL and B-ALL persists as the antibody is diluted 4-fold (to 1:1,000) and 10-fold (to 1:2,500).
and HCL-V from an activated B cell at a late stage of maturation.16 CD200 appears to be able to distinguish among these post-germinal center B-cell–derived neoplasms: HCL is consistently strongly positive for CD200 by flow cytometric and flow cytometric analysis; HCL-V exhibits low-level CD200 expression, and MZL and LPL exhibit variable CD200 expression. This is in contrast to T-bet, for example, a T-cell transcription factor that we found to be consistently expressed in a number of B-cell lymphoproliferative disorders, including CLL/SLL, MZL, and HCL,18 as well as in HCL-V, as shown in the current study. The difference in CD200 expression by HCL and HCL-V provides new evidence that these B-cell lymphoproliferative disorders are biologically distinct. Similarly, T-bet was expressed in classic Hodgkin lymphoma as well as nodular lymphocyte-predominant Hodgkin lymphoma, while CD200 was expressed in the former but not in the latter neoplasm.11,19

Recently, CD1d was reported as a flow cytometric marker useful for the differential diagnosis of B-cell lymphoproliferative disorders.20 CLL/SLL was mostly negative for CD1d; MCL, LPL, and HCL-V exhibited low-level CD1d expression; MZL exhibited higher-level CD1d expression; and HCL exhibited the highest level of CD1d expression. Consequently, CD1d staining may be useful to distinguish MZL from HCL-V and LPL.20 Another flow cytometric study of CD103-positive B-cell lymphoproliferative disorders found that CD25 and annexin A1 expression may be useful to distinguish HCL, which expresses CD25 and annexin A1, from HCL-V and splenic marginal zone lymphoma, which are negative for these antigens but may be CD103 positive.21 The use of one of the above markers alone may be insufficient for definitive classification of B-cell lymphoproliferative disorders, but a flow cytometric pattern that incorporates CD1d, CD103, CD25, and CD200 should be useful for differential diagnosis and subtyping of CD5-negative, CD10-negative small B-cell lymphoproliferative disorders: HCL is strongly positive for CD1d and CD200 and also expresses CD103 and CD25. In contrast, HCL-V, although positive for CD1d, is negative for CD25 and CD200 and exhibits low-level CD1d expression. SMZL, which may be positive for CD25, is negative for CD103 and CD200 and positive for CD1d.

The relative staining intensity of CD200 as determined by flow cytometric analysis is also useful in the differential diagnosis of other B-cell lymphoproliferative disorders. CLL/SLL exhibits strong staining for CD200 in contrast to MCL, which is CD200 negative. This is consistent with the findings of Palumbo and coworkers,22 who found that CD200 is expressed in CLL/SLL and not in MCL, based on flow cytometric immunophenotyping as well as immunohistochemical staining of clinical samples, our prior immunohistochemical findings,11 and more recent flow cytometric findings reported by Alapat and coworkers.23 Similarly, B-ALL exhibits strong staining for CD200 in contrast to BL, as we previously observed by immunohistochemical staining.11 In general, flow cytometric immunophenotypic analysis for CD200 substantiates immunohistochemical staining results for CLL, MCL, FL, B-ALL, HCL, and BL.11

Recently, anti-CD200 targeted therapy has been proposed as a treatment for CD200-positive neoplasms, including CLL/SLL. In an animal model of CLL, anti-CD200 antibody administration resulted in nearly complete tumor growth inhibition.10 The effect of anti-CD200 antibody in this animal model is thought to be due to blocking of CD200 receptor-ligand interaction as well as elimination of activated T cells involved in immune-mediated killing of neoplastic cells.10,24,25 ALXN6000, a humanized, murine-derived anti–human CD200 antibody, is in a clinical trial as a potential novel immunotherapeutic agent to treat CLL/SLL and multiple myeloma.25 (NCT00648739). If such therapy is found to be efficacious for CD200-positive neoplasms such as CLL/SLL, it may be suitable to treat other CD200-positive B-cell–derived neoplasms, particularly HCL and B-ALL, which, along with CLL/SLL, exhibit high levels of CD200 expression.

References