Diagnostic Application of Two-Color Flow Cytometry in 161 Cases of Hairy Cell Leukemia

By Bruce A. Robbins, Douglas J. Ellison, John C. Spinosa, Cristin A. Carey, Robert J. Lukes, Sibrand Poppema, Alan Saven, and Lawrence D. Piro

Recent immunophenotypic studies of hairy cell leukemia (HCL) have suggested specific patterns of immunoreactivity that may aid in diagnosis. We studied peripheral blood (PB) from 161 cases of HCL using two-color direct immunofluorescence flow cytometry and an extended panel of antibody combinations. Circulating hairy cells were identified by immunophenotypic features in 92% of the cases and could be detected even when representing ≤1% of circulating lymphocytes. The 133 cases with ≥2% detectable hairy cells were analyzed in detail. HCL showed a uniform and unique B-cell phenotype, with each of the following features identified in 99% to 100% of cases: (1) positive staining for CD19, coexpressed with CD20; (2) very intense, uniform expression of CD11c, with CD19; (3) moderately intense staining for CD22, with CD19; (4) very intense staining for CD22; (5) moderate to very intense staining for CD20; and (6) moderately intense monoclonal surface Ig. Phenotypic variability existed in expression of CD10 (26%) and CD5 (4%). Based on these features, HCL was easily distinguished from 50 cases of chronic lymphocytic leukemia (CLL). Although CLL exhibited frequent expression of CD11c (74%) and CD25 (68%), the intensity of staining was significantly less than HCL. Furthermore, CLL was uniformly positive for CD5 and showed weak staining for CD20, CD22, and surface Ig. B-ly7 proved to be the most specific marker, reacting with 100% of HCL cases, but absent in all cases of CLL. We conclude that two-color flow cytometry with specific antibody combinations is an efficacious method for characterization and sensitive detection of hairy cells in PB. Application of the phenotypic criteria described should help to increase accuracy in diagnosis of HCL.

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Hairy Cell Leukemia (HCL) is a rare but well-recognized lymphoproliferative disorder (LPD) that is characterized by splenomegaly, pancytopenia, bone marrow (BM) infiltration, and circulating leukemic cells with cytoplasmic projections. The standard approach to diagnosis of HCL involves examination of peripheral blood (PB) and BM, with identification of the distinctive morphologic features of hairy cells and demonstration of strong cytoplasmic staining for tartrate-resistant acid phosphatase (TRAP). However, difficulty may arise in distinguishing HCL from other unusual LPDs (eg, splenic lymphoma with villous lymphocytes, monocytoid B-cell lymphoma, and prolymphocytic leukemia), and from morphologic variants of more common LPD's such as chronic lymphocytic leukemia (CLL) and low-grade non-Hodgkin's lymphomas (NHL). Additionally, LPD's other than HCL may show weak to moderate TRAP staining.

Immunophenotypic analysis may significantly aid in the diagnosis of HCL. Previous studies have shown HCL to be a B-cell neoplasm with some unusual phenotypic features. In addition to monoclonal surface Ig and pan B-cell markers (CD19, CD20, and CD22), HCL expresses CD11c, a marker primarily associated with myelomonocytic cells, and CD25, the interleukin-2 (IL-2) receptor. Although the presence of these markers is not specific for HCL, there is evidence that a pattern of intense expression of CD11c and CD22 may be a unique finding. In recent reports, monoclonal antibody (MoAb) B-ly7, and related antibodies HML-1, Ber-ACT8, and LF61 have shown high specificity for HCL when coexpressed with a pan B-cell marker. Two-color flow cytometry has been proposed as an optimal method to identify the unique immunophenotypic profiles in HCL. However, this methodology has been studied only with selected antibodies in relatively small numbers of HCL cases, and has not been comprehensively evaluated.

We report 161 cases of HCL analyzed by two-color direct immunofluorescence flow cytometry using a large panel of monoclonal and polyclonal antibody reagents. The goals of this study were to: (1) confirm and extend previous observations on immunophenotypic characteristics of HCL; (2) identify antibody combinations and patterns of two-color immunofluorescence staining that are most useful in the diagnosis of HCL; (3) measure fluorescence intensity to objectively confirm the observed patterns of immunofluorescence staining; and (4) compare the immunophenotypic features of HCL with CLL and other B-cell LPD's.

MATERIALS AND METHODS

Case material. All patients included in this study were evaluated at Scripps Clinic and Research Foundation, La Jolla, CA, between May 1, 1990 and August 1, 1992, for treatment with 2-chlorodeoxyadenosine (2-CdA). Diagnoses of typical HCL were confirmed in all cases by standard morphologic criteria, with examination of BM, PB, and TRAP stains. Atypical morphologic variants of HCL were excluded from this study. HCL patients were either previously untreated, or had undergone prior splenectomy and/or treatment with α-interferon or 2-deoxycoformycin. Cases of CLL were confirmed by standard morphologic criteria. Patients with CLL had undergone previous alkylator-containing chemotherapy. Normal control cells were obtained from healthy adult volunteers. Approval was obtained from our Institutional Review Board for

From the Department of Pathology, and the Division of Hematology and Oncology, Scripps Clinic and Research Foundation, La Jolla, CA, and the Department of Laboratory Medicine, Cross Cancer Institute, Edmonton, Alberta, Canada.

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Address reprint requests to Bruce A. Robbins, MD, Department of Pathology, Scripps Clinic and Research Foundation, 10666 N Torrey Pines Rd, La Jolla, CA 92037.

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these studies. Written informed consent was obtained from all patients.

Blood specimens. Fifty milliliters of PB were collected into acid-citrate-dextrose (ACD-A)-containing vacutainer tubes. The blood samples were held at room temperature no longer than 24 hours before processing.

Mononuclear cell isolation. PB mononuclear cells were isolated by centrifugation of Buffy coat over a Ficoll-Hypaque density gradient. The isolated mononuclear cells were washed twice in RPMI 1640.

The first 85 cases of HCL were viably cryopreserved by slow freezing in RPMI containing 10% dimethyl sulfoxide (DMSO) and 10% fetal calf serum (FCS). These viably frozen cells were then maintained in liquid nitrogen. When later thawed for analysis, the cells were washed twice in RPMI 1640 containing 10% FCS.

Antibody reagents. The MoAb reagents used are listed in Table 1. Affinity-purified goat polyclonal antibodies were used to detect Igm, IgD, K, and L. lambda and k reagents from two different sources were used to confirm results of light-chain typing. Each antibody reagent was directly conjugated with either fluorescein isothiocyanate (FITC) or phycoerythrin (PE).

Immunofluorescence staining. A two-color direct immunofluorescence staining technique was used. Before staining, viability of the mononuclear cell preparation was determined by trypan blue dye exclusion; viability in excess of 90% was required. The cells were washed and resuspended at a concentration of 2 x 10^6 cells/mL in phosphate-buffered saline (PBS) with 10% heat-inactivated rabbit serum. This was followed by incubation at 37°C for 30 minutes to shed cytophilic Ig. Fifty microliters of cell suspension (1 x 10^6 cells) was then added to each of 15 tubes, which received a designated amount of paired FITC- and PE-conjugated antibodies (Table 2). The cells were subsequently incubated with antibody in an ice bath, in the dark for 30 minutes, washed twice with PBS, and resuspended in 0.5 mL of 1% paraformaldehyde.

Flow cytometric analysis. The stained cells were analyzed in a Becton Dickinson FACScan flow cytometer (Becton Dickinson, San Jose, CA) using Consort 30 software. Ten thousand events were acquired using log amplification for green and red fluorescence. Cells were displayed on a forward versus right angle light scatter plot, and appropriate gating of the lymphocyte population was confirmed by immunophenotypic identification of lymphocytes versus monocytes on the CD45/CD14 tube. Routine gating of HCL specimens included the small lymphocyte population and always extended into the large lymphocyte and monocyte regions, to encompass the zone where hairy cells are concentrated (Fig 1). In some cases, additional analysis was performed by gating only on the large cell region. This large cell subgating procedure effectively analyzed a concentrated population of hairy cells, and excluded the predominant population of reactive small B and T lymphocytes. Large cell subgating was especially useful in cases with low numbers of hairy cells to show monoclonal light chain restriction and other phenotypic features of HCL in the appropriate light scatter region.

The quadrant markers designating positivity for FITC or PE were drawn according to the position of the negative population on the isotype control plot. A cell population was considered positive for a given surface marker if more than 20% of the cell population stained positively. Mean fluorescence intensity (MFI) of a positive population of cells was calculated by the Consort 30 software. Throughout the study, instrument performance was monitored according to standard quality assurance procedures including daily analysis of fluorescent bead standards. No significant fluctuations in fluorescence detection were identified. The instrument gain settings for green and red fluorescence remained constant throughout the study.

RESULTS

On analysis of PB in 161 cases of HCL, circulating hairy cells were identified by immunophenotypic features in 148
cases (92%). The level of detectable circulating hairy cells varied widely, from less than 1% to more than 95% of total lymphocytes. The absolute lymphocyte count varied from 300/μL to 74,900/μL, with a median of 1,800/μL. The absolute B-cell (CD19) count ranged from less than 10/μL up to 63,700/μL, with a median of 340/μL. Circulating hairy cells were detectable in all cases with absolute CD19 B-cell counts greater than 170/μL. There were 15 cases (9%) with less than 2% detectable hairy cells; because of the difficulty with complete phenotypic characterization of very small numbers of hairy cells, and concerns about statistical accuracy of MFI determinations, these cases were excluded from further analysis. The 133 cases (83%) with immunophenotypically confirmed HCL comprising ≥2% of the lymphocyte population served as the basis for further detailed analysis.

In cases with greater than 10% to 15% leukemic cells, the HCL population was identifiable on the forward versus right-angle light scatter plot as a distinct population of large cells that overlapped with the region in which monocytes are normally found (Fig 1). Accurate evaluation required inclusion of this large cell population in the region gated for analysis. The HCL cases typically exhibited prominent monocytopenia, with ≤1% monocytes (CD14+/CD45+) in the gated region. Monocytopenia and small numbers of large reactive lymphocytes in the gated region were easily distinguished from hairy cells by phenotypic features.

HCL showed a very consistent immunophenotype (Table 3). All cases of HCL expressed the pan B-cell markers, CD19, CD20, and CD22. Monoclonal surface Ig was identified in 99% (132 of 133) of the HCL cases analyzed. In some cases with small numbers of hairy cells, it was difficult to define monoclonality with the routine gating procedure because of the admixture with normal polyclonal B cells. Most of these cases could be resolved by specific subgating on the large cell region, which focused the analysis on the HCL population, and excluded the small lymphocyte population where the reactive B cells are predominantly localized (Fig 2). Ig heavy chain expression included coexpression of IgM and IgD in 48% (64 of 133), IgM alone in 41% (54 of 133), and undetectable IgM or IgD in 11% (15 of 133). IgG and IgA were not analyzed. There was more frequent expression of μ light chain (54%) than κ light chain (45%). One-hundred percent of the HCL cases exhibited coexpression of B-ly7 with CD19, and CD11c with CD19. CD25 coexpression with CD19 was present in 99% (132 of 133) of HCL cases.

On two-color contour plots, hairy cells were identified as a discrete population of cells with characteristic patterns of immunofluorescence staining (Fig 3). The distinctive features included: (1) positive staining for B-ly7 with CD19; (2) very intense, uniform staining for CD11c, coexpressed with CD19; (3) moderately intense, relatively uniform staining for CD25 with CD19; (4) very intense staining for CD22; (5) moderate to very intense staining for CD20; (6) CD5 negativity, with rare exceptions; and (7) moderately intense monoclonal surface Ig (Fig 2).

Some variability of immunophenotypic expression was found. Five cases of HCL (4%) were identified with positive staining for CD5, which generally appeared weak and nonuniform. CD10 expression was identified in 26% (34 of 133) of the HCL cases. We observed a consistent property of CD20 antibody to cause aggregation of hairy cells. Because the aggregated cells

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**Table 3. Results of Phenotypic Analysis**

<table>
<thead>
<tr>
<th></th>
<th>HCL (n = 133)</th>
<th>CLL (n = 50)</th>
<th>P Value*</th>
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<tbody>
<tr>
<td>CD19</td>
<td>133 (100)</td>
<td>50 (100)</td>
<td>NS</td>
</tr>
<tr>
<td>CD20</td>
<td>133 (100)</td>
<td>49 (98)</td>
<td>NS</td>
</tr>
<tr>
<td>CD22</td>
<td>133 (100)</td>
<td>47 (94)</td>
<td>.02</td>
</tr>
<tr>
<td>IgM + IgD</td>
<td>64 (48)</td>
<td>34 (68)</td>
<td>.02</td>
</tr>
<tr>
<td>IgM</td>
<td>54 (41)</td>
<td>5 (10)</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>IgM/IgD</td>
<td>15 (11)</td>
<td>11 (22)</td>
<td>NS</td>
</tr>
<tr>
<td>λ</td>
<td>60 (45)</td>
<td>29 (58)</td>
<td>NS</td>
</tr>
<tr>
<td>λ/κ</td>
<td>1 (1)</td>
<td>8 (16)</td>
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</tr>
<tr>
<td>CD11c</td>
<td>133 (100)</td>
<td>37 (74)</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>CD25</td>
<td>132 (99)</td>
<td>34 (68)</td>
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</tr>
<tr>
<td>B-ly7</td>
<td>133 (100)</td>
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</tr>
<tr>
<td>CD3</td>
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<td>NS</td>
</tr>
<tr>
<td>CD6</td>
<td>5 (4)</td>
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</tr>
<tr>
<td>CD10</td>
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<tr>
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<td>0 (0)</td>
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<td>NS</td>
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<tr>
<td>CD16</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>NS</td>
</tr>
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</table>

Percentages are in parentheses.
Abbreviation: NS, not significant.
* Fisher's exact test.

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**Fig 1.** Light scatter plot in HCL. Hairy cells (H) are recognized as a discrete population of large cells, with higher forward (FSC) and side (SSC) angle light scatter than normal lymphocytes (L). Gating for analysis must include the large cell region, as shown.
Fig 2. A case of HCL in which specific gating on the large cell region (A) identifies a small population of hairy cells with intense CD11c/CD19 coexpression (B), and monoclonal κ surface Ig (C and D). Light chain restriction could not be shown on routine gating because of dilution by normal polyclonal B lymphocytes. Hairy cells represented 4% of PB lymphocytes in this case.

are not included in the cell population gated for analysis, determinations of percent CD20+ cells were uniformly lower than those of CD19 or CD22. This phenomenon was not observed in CLL.

In the 50 cases of CLL studied, coexpression of CD11c with CD19 was observed in 74% (37 of 50), and expression of CD25 with CD19 detected in 68% (34 of 50). None of the CLL cases stained positively for B-ly7. As expected, all 50 cases of CLL were positive for CD5. Although CD11c and CD25 were identified in a substantial proportion of CLL,

Fig 3. Characteristic immunophenotypic features of HCL shown on two-color contour plots: (A) positive staining for B-ly7, coexpressed with CD19; (B) very intense, uniform expression of CD11c, with CD19; (C) moderately intense staining for CD25, with CD19; (D) very intense staining for CD22 (this case is negative for CD10); (E) intense expression of CD20; and (F) absence of staining for CD5.
the patterns of immunofluorescence staining on two-color contour plots were distinct from HCL (Fig 4). CLL cells showed only weak to moderate expression of CD11c or CD25, often exhibiting a broad range of fluorescence intensity from negative to positive within the leukemic cell population. Other features characteristic of CLL included weak staining for CD20, CD22, and surface Ig.

The different patterns of immunofluorescence staining in HCL and CLL were further analyzed by quantitation of MFI for each of the surface markers studied (Table 4). The MFI measurements confirm the visual impression of significantly brighter staining for CD20, CD22, CD11c, and CD25 in HCL compared with CLL (P < 0.0001). This analysis also shows stronger expression of CD19, IgD, κ, and λ in HCL than CLL. The fluorescence intensity of surface IgM was comparable in HCL and CLL, but decreased when compared with normal B lymphocytes. The intensity of CD19, CD20, and CD22 expression was increased in HCL compared with normal control B-cells, whereas expression of IgD, κ, and λ was nearly equivalent.

Less than 3% of normal lymphocytes were CD11c+ / CD19+ or CD25+/CD19+, and ≤1% of lymphocytes were B-ly7+/CD19+. On two-color contour plots, these represented a few scattered cells with low fluorescence intensity (Fig 5). Cases of HCL with small numbers of circulating hairy cells could be distinguished by demonstration of a discrete population of cells with stronger and more uniform staining for CD11c, CD25, and B-ly7 than normal lymphocytes.

**DISCUSSION**

Accurate diagnosis of HCL has become critically important with the development of increasingly effective therapeutic agents (α-interferon, deoxycoformycin, 2-CdA) able to induce responses in a high percentage of patients. However, most pathologists and hematologists have limited experience with this rare leukemia, and differential diagnosis with the use of current flow cytometry instrumentation and PE-conjugated CD11c antibody. Despite the frequent expression of CD11c by CLL, HCL can be readily distinguished by its nearly 30-fold higher intensity of CD11c expression. This differentiation is easily made by direct visual inspection of the CD11c/CD19 two-color histograms (Figs 3B and 4B). Our observations are very similar to those of Miller, et al who concluded that a pattern of uniformly intense coexpression of CD11c and CD22 was unique to HCL, and not encountered in other B-cell LPD's.

HCL was the first B-cell LPD recognized to express the IL-2 receptor, CD25. Subsequent studies have shown expression of CD25 in up to 50% of various B-cell NHLs, and in 40% of B-cell CLL. Our observations of CD25 expression in 99% of HCL cases and 66% of CLL further show that positivity for CD25, while characteristic of HCL, is not specific. However, as we have shown, the fluorescence intensity of CD25 staining in HCL averages sixfold higher than CLL, and this can be a useful discriminating factor.

MoAb B-ly7 was developed by Visser et al in 1989 in an effort to develop a useful diagnostic and investigative reagent against HCL. In a study of 150 samples of various B-cell lymphoproliferative diseases, B-ly7 was shown to have very high sensitivity and specificity for HCL. The antibody reacted with all 28 cases of HCL examined, but was negative in all cases of CLL, prolymphocytic leukemia, and B-cell NHL, including splenic lymphoma with villous lymphocytes. Subsequent studies have produced similar results. Our data confirm the diagnostic value of B-ly7, which reacted with 100% of the HCL cases, and none of the CLL cases. Our additional experience with over 50 cases of various B-cell NHL has shown no reactivity for B-ly7. In our experience, coexpression of B-ly7 with CD19 was the marker combination most specific for HCL.

**Fig 4.** Characteristic immunofluorescent staining patterns in CLL expressing CD11c and CD25. In contrast to HCL: (A) B-ly7 is negative; (B) CD11c is weakly staining, with a broad range of reactivity; (C) CD25 staining is weak; (D) CD22 expression is very weak; (E) CD20 staining is weak; and (F) CD5 is positive.
Recently, it was recognized that B-ly7 has identical reactivity to MoAb HML-I, previously described as a marker of intestinal intra-epithelial lymphocytes. Two additional MoAb, Ber-ACT8 and LF6, have been shown to have identical specificity. These antibodies recognize an antigen expressed on intraepithelial T lymphocytes, small subpopulations (<1%) of PB B and T cells, activated monocytes, hairy cells, and some T-cell lymphomas, indicating enteropathy-associated T-cell lymphomas. The antigen has recently been identified as a subunit of a β7 integrin molecule, which may play a role in lymphocyte homing and adhesion.

B-cell antibody CD22 has been reported to produce significantly higher staining intensity in hairy cells than in either normal B-cells or leukemic cells from other B-cell LPDs. Our study confirms these observations by demonstrating mean fluorescence intensity of CD22 in hairy cells to be over five times higher than normal B lymphocytes, and almost 50 times higher than CLL, which is known to have very weak expression of CD22. We also observed bright fluorescence for CD20 to be characteristic of HCL. This contrasted with weak CD20 expression in CLL, as has been previously reported. These findings are diagnostically important and indicate the need to include evaluation of staining intensity for CD22 and CD20 as part of the phenotypic analysis for HCL.

Expression of CD5 is a well-recognized feature of B-cell CLL and was present in 100% of our CLL cases. However, we did identify five cases (4%) of HCL that were positive for CD5. CD5+ HCL has previously been documented, although there is disagreement on the frequency of its occurrence. Two studies have reported CD5 expression in 20% to 30% of HCL, whereas most other studies have shown this to be an unusual finding. In one of the former studies, CD5 expression was found to predict a poor response to α-interferon therapy. The reported occurrence of CD5 in HCL and CD11c in CLL has generated discussion about the relationship of these leukemias. In vitro studies have shown induction of HCL-like morphology, TRAP positivity, and expression of HCL-associated surface markers (CD11c and CD25, but not B-ly7) by incubation of CLL cells with phorbol esters. Rare cases have been reported that appear to have combined features of HCL and CLL. However, these reports are difficult to interpret in view of the limited specificity of positive staining for CD11c, CD25, CD5, and TRAP. It is conceivable that many cases previously reported as CD5+ HCL, or as HCL/CLL hybrids, may represent variants of CLL with weak CD11c and TRAP positivity. On the other hand, the presence of CD5 positivity cannot be used to exclude a diagnosis of HCL. A complete immunophenotypic analysis, including evaluation for B-ly7, intensity of CD11c staining, and other specific patterns of reactivity would help to resolve these cases, and perhaps define new entities.

Although HCL is now well established as a B-cell leukemia, a normal counterpart to the hairy cell in B-cell ontogeny remains undefined. Most investigators agree that HCL is at a late stage of B-cell differentiation, based on studies showing absence of CD21, frequent expression of IgG and IgA, and expression of early plasma cell marker PCA-1. This contrasts with CLL, which is believed to derive from an earlier stage of B-cell development, and whose normal counterpart is most likely the CD5+ B cell. We were unable to identify any normal PB B-cell population exhibiting the phenotypic profile of HCL. If a normal counterpart to the hairy cell exists, it may be a very rare cell type, possibly a subset of B-ly7+ B cells.

We conclude that two-color flow cytometry is a useful adjunctive method, which, in combination with routine morphologic and TRAP staining, should significantly enhance the accuracy in diagnosis of HCL. Application of a complete antibody panel is recommended over the use of any single antibody marker, because recognition of the total phenotypic profile provides the highest level of specificity and sensitivity. Immunohistochemical techniques on BM biopsies have shown residual hairy cells in some cases that were not identified by conventional morphologic examination. Application of flow cytometry for this purpose has not been as extensively studied. However, the high sensitivity of two-color flow cytometry suggests that this

<table>
<thead>
<tr>
<th>Table 4. MFI Measurements of Positive Antibody Staining</th>
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<tr>
<td>HCL*</td>
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<td>------</td>
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<tr>
<td>MFI (±1 SD)</td>
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<tr>
<td>CD19</td>
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<td>CD20</td>
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<td>CD22</td>
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<td>IgM</td>
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<td>λ</td>
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<tr>
<td>CD11c</td>
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<tr>
<td>CD25</td>
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<tr>
<td>B-ly7</td>
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* This analysis includes all cases of HCL or CLL positive for each listed antibody. The corresponding numbers of cases appear in Table 3.
† B-ly7 was not included in this analysis, because there was no positive staining of CLL or normal controls to compare with HCL.

<table>
<thead>
<tr>
<th>Table 5. Summary of Typical Immunophenotype in HCL and CLL</th>
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<tbody>
<tr>
<td>HCL</td>
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<tr>
<td>------</td>
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<tr>
<td>CD19</td>
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<tr>
<td>CD20</td>
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<tr>
<td>CD22</td>
</tr>
<tr>
<td>Surface Ig</td>
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<tr>
<td>CD6</td>
</tr>
<tr>
<td>CD11c</td>
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<tr>
<td>CD25</td>
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<tr>
<td>B-ly7</td>
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Abbreviations: --, negative; + to ++++, weak to very strong positive staining.
* Rare cases of HCL are CD5+.
† A minority of CLL is CD11c- and/or CD25-.
FLOW CYTOMETRY IN Hairy Cell Leukemia

would be an ideal method for posttreatment monitoring of PB or BM. Studies are currently under way to document the efficacy of this approach.

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Fig 5. Normal control lymphocytes. Rare CD19+ B cells coexpress B-ly7 (A), CD11c (B), or CD25 (C), with low staining intensity.
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