A Two-Color Flow Cytometry Assay for Detection of Hairy Cells Using Monoclonal Antibodies

By Jørgen Schøler Kristensen, Jørgen Elleegaard, and Peter Hokland

We have developed a simple two-color immunofluorescence assay equally suited for microscopy and flow cytometry detecting hairy cells (HCs) in single cell suspensions, based on the concomitant reactivities with the B cell-specific monoclonal antibody B1 (CD20) and the monocyte/HC-associated antibody SHCL-3 (CD11c). Thus, HCs can be demonstrated in peripheral blood, bone marrow, and spleen specimens from hairy cell leukemia (HCL) patients even when they constitute <1% of the cell suspension. Likewise, admixture experiments with normal patients even when they constitute <1% of the cell suspension. Likewise, admixture experiments with normal patients even when they constitute <1% of the cell suspension. Thus, HCs can be demonstrated in peripheral blood, bone marrow, and spleen specimens from hairy cell leukemia (HCL) patients even when they constitute <1% of the cell suspension.

Hairy cell leukemia (HCL) is recognized to be of B cell origin due to the presence of B cell-restricted surface determinants and by the rearrangement of immunoglobulin genes in HCs. Furthermore, based on the reactivity of HCs with plasma cell-associated antibody PCA-1, HCL has been suggested to be a pre-plasma cell tumor. In line with this observation, recent evidence using B cell differentiation stimuli has suggested that the normal counterpart of HCs might be an activated B-lymphoblast. The other hand, HCs can express antigens normally associated with myelomonocytic cells, especially the Mo1 antigen, which has been found on most but not all HCL samples. The functional significance of the presence of these myeloid antigens on a purported B cell tumor is at present unknown.

Because HCL is a well-established clinicopathologic entity, its diagnosis and longitudinal monitoring of the disease have proven difficult, partly because the number of circulating HCs can be low and partly because the morphology and tartrate-resistant acid phosphatase (TRAP) staining are not necessarily reliable diagnostic parameters. Furthermore, since none of the monoclonal antibodies (Mabs) yet published have shown specificity for HCs, immunologic identification of these cells is based on the successive reactivities with different Mabs. Because no other hematopoietic cells to our knowledge have shown similar evidence of simultaneous expression of myelomonocytic and B cell-associated antigens, we hypothesized that an assay identifying both such differentiation antigens concomitantly on the single cell level might be an optimal way of identifying HCs. Here we present data on such a test using the B cell-specific B1 antibody and the monocytic SHCL-3 antibody. HCs could be definitively identified and purified even when they constituted <1% of cell suspensions.

MATERIALS AND METHODS

Patients. Samples from 18 patients with clinically and morphologically verified HCL were obtained after informed consent was given. No patients had received cytostatic treatment prior to sampling.

Isolation of mononuclear cells. Mononuclear cells (MNCs) were obtained from peripheral blood by Ficoll-Hypaque density centrifugation. MNC samples from spleens were obtained after mincing freshly obtained tissue with forceps and scissors, followed by repeated suction in a 21-gauge needle. Samples were labeled bright red, whereas the SHCL-3 antibody binding sites were labeled bright green. After a 30-minute incubation period at 4°C, the cells were washed three times in PBS-AB and analyzed as described below.

Two-color fluorescence labeling of HCL samples. One million cells in 100 μL were incubated with approximately diluted (in phosphate-buffered saline (PBS) with 2.5% human AB-serum (PBS-AB)) ascites containing the Mabs at 4°C for 30 minutes. Reactivities and sources of the antibodies used are shown in Table 1. After two washes in PBS-AB, the cells were incubated with a fluorescein (FITC)-conjugated F(ab) rabbit anti-mouse IgG (R/M-Ig FITC, Dakopatts, Copenhagen). After a 30-minute incubation period at 4°C, the cells were washed three times in PBS-AB and analyzed as described below.

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Normal rabbit serum conjugated with FITC was used as a secondary layer control (Dakopatts).

Flow cytometry analysis. Antibody-labeled cells were analyzed by flow cytometry in an EPICS C flow cytometer (FCM) (Coulter) after appropriate forward-lightscatter gating. For each sample, at least 10,000 cells were analyzed using the log amplification mode, with negative controls being MNCs labeled with second antibody layer alone. The intensity of cell surface fluorescence was estimated by the mean channel number of the log-fluorescence histogram. Double-labeled cell suspensions were analyzed after appropriate forward-lightscatter gating and optimization of the filters to PE and FITC using the two-parameter histogram. The number of double-labeled cells was calculated using the QuadStat statistics program of the EPICS C. For optimization purposes, the flow cytometer was adjusted daily using green and red fluorescent calibration beads (Coulter Electronics, Luton, England).

RESULTS

Reactivity of HCs with various myeloid and lymphoid antibodies. In the process of phenotyping blood, bone marrow, and spleen specimens from HCL patients we noticed a marked heterogeneity in the expression of most of the leukocyte differentiation antigens detected by Mabs both with respect to the number of positive cells and to the intensity of the antigens detected. As is shown in Table 2, the reactivity with an antibody against a myeloid differentiation antigen (eg, My7) on homogenous HCL spleen suspensions could vary between 1% and 86% of positive cells. The same was true for the My8, My9, Mo1, and Mo2 antigens. In contrast, the monocytic antibody My4 consistently reacted with most of the HCs, albeit with low intensity in some instances. The SHCL-3 antibody was present in high density of these B cell-associated antigens, it is noteworthy that whereas the common ALL antigen (CALLA) (J5), B4, and light chains were only slightly expressed, the B1 antigen was always present in high density.

Two-color fluorescence detection of HCs using SHCL-3 and B1. Although both SHCL-3 and B1 are highly expressed on HCs, their expression on monocytes and B cells, respectively, make their use as single reagents for the detection of HCs impossible. With the introduction of PE-avidin conjugates and the availability of biotin-conjugated Mabs, however, it has become possible to perform two-color studies both with the fluorescence microscope as well as with single-laser flow cytometers since FITC and PE have different emission spectra but share a common excitation wavelength (488 nm). Because both B1 and SHCL-3 react with HCs, we designed a dual-color fluorescence test using SHCL-3 + R/M-Ig FITC and B1-avidin + avidin-PE. In the fluorescence

Table 1. Monoclonal Antibodies Used in This Study

<table>
<thead>
<tr>
<th>CD*</th>
<th>Mab Used</th>
<th>Reactivity</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>UCHT-1</td>
<td>Mature T cells</td>
<td>B. Perussia</td>
</tr>
<tr>
<td>10</td>
<td>J5</td>
<td>CALLA</td>
<td>J. Ritz</td>
</tr>
<tr>
<td>11b</td>
<td>MO1</td>
<td>C3bi receptor</td>
<td>J. D. Griffin</td>
</tr>
<tr>
<td>13</td>
<td>My7</td>
<td>Immature myeloid</td>
<td>J. D. Griffin</td>
</tr>
<tr>
<td>14</td>
<td>My4, Mo2</td>
<td>Monocytes</td>
<td>J. D. Griffin</td>
</tr>
<tr>
<td>11c</td>
<td>SHCL-3</td>
<td>LFA + gp 150/95</td>
<td>R. Schwarting</td>
</tr>
<tr>
<td>19</td>
<td>B4</td>
<td>B cells</td>
<td>L. M. Nadler</td>
</tr>
<tr>
<td>20</td>
<td>B1</td>
<td>B cells</td>
<td>L. M. Nadler</td>
</tr>
<tr>
<td>21</td>
<td>B2</td>
<td>C3d receptor</td>
<td>L. M. Nadler</td>
</tr>
<tr>
<td>33</td>
<td>My9</td>
<td>Immature myeloid</td>
<td>J. D. Griffin</td>
</tr>
<tr>
<td>None</td>
<td>la</td>
<td>HLA-DR antigen</td>
<td>L. M. Nadler</td>
</tr>
</tbody>
</table>

*Cluster of differentiation defined by the International Leucocyte Typing Workshops (Oxford revision, 1986).
†Leucocyte Function Antigen. (For a thorough review of these reagents, see A. McMichael, et al, eds: Leucocyte Typing Workshop III, Oxford University Press, 1986.)

Table 2. Analysis of Surface Marker Antigens on HC Suspensions

<table>
<thead>
<tr>
<th>Mab</th>
<th>Spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 2 3 4 5 6</td>
</tr>
<tr>
<td>My4</td>
<td>90 90 76 72 56 54</td>
</tr>
<tr>
<td>My7</td>
<td>&lt;1 81 86 17 15 26</td>
</tr>
<tr>
<td>My8</td>
<td>&lt;1 &lt;5 17 &lt;5 &lt;5 &lt;5</td>
</tr>
<tr>
<td>My9</td>
<td>83 14 14 23 5 21</td>
</tr>
<tr>
<td>MO-1</td>
<td>64 9 26 40 34 49</td>
</tr>
<tr>
<td>MO-2</td>
<td>21 75 43 &lt;2 &lt;2 7</td>
</tr>
<tr>
<td>J5</td>
<td>82 87 81 10 11 53</td>
</tr>
<tr>
<td>B1</td>
<td>91 &gt;95 &gt;95 61 72 52</td>
</tr>
<tr>
<td>B2</td>
<td>12 16 11 &lt;2 11 &lt;5</td>
</tr>
<tr>
<td>B4</td>
<td>78 &gt;95 27 80 76 63</td>
</tr>
<tr>
<td>la</td>
<td>90 95 95 80 90 67</td>
</tr>
<tr>
<td>UCHT1</td>
<td>8 3 2 20 2 26</td>
</tr>
<tr>
<td>SHCL-3</td>
<td>80 92 89 63 85 74</td>
</tr>
<tr>
<td>SHCL-3/B1</td>
<td>91 95 94 56 76 58</td>
</tr>
<tr>
<td>B1-BIO</td>
<td>76 81 85 69 68 54</td>
</tr>
</tbody>
</table>

*Percentage of positive cells.
†Dominating light chain.

Fig 1. Hairy cells from peripheral blood with 71% TRAP+ cells identified by two-color flow cytometry. Two parameter dot plot of integrated SHCL-3 (x-axis) B1 (y-axis) signals analysed by Quadsat statistics showed 61% double-marker-positive cells. Negative controls revealed 0.5% to 1.3% double-marker-positive cells.
In double-labeling cases of: (a) histograms showing the results identified. Ble-marker-positive cells were B1 + demonstrated. As compared with the microscope reading, SHCL-3+/B1 and from I 2 with chronic lymphocytic leukemia (CLL) that suited controls. Thus, as is shown in Table 3, cells from five assay. Thus, negative controls including relevant second-layer antibodies only and first-layer Mabs of similar isotype when compared with TRAP staining in 18 samples from nine patients, correlation between the double-marker assay and TRAP, a surprising heterogeneity of reactivity patterns was observed. When the number of SHCL-3/B1-positive cells was compared with TRAP staining in 18 samples from nine patients, a surprising heterogeneity of reactivity patterns was observed (Fig 5). Thus, although some of the samples showed a close correlation between the double-marker assay and TRAP, two patients were 60% to 82% SHCL-3/B1 positive but TRAP negative. When evaluated on Giemsa-stained smears, one of the patients had >60% circulating HCs, the other patient showed only abnormal MNCs with some indications of HCs. Diagnosis was made on bone core biopsy. Thus, these patients demonstrate the value of the immunologic assay when TRAP staining is negative or when the HC morphology is atypical.

To elucidate the clinical value of this assay further, we

\begin{table}
\centering
\begin{tabular}{lccc}
\hline
Source & Mab & \\
\hline
Normal donor MNCs (25) & B1 & SHCL-3 & SHCL-3/B1 \\
B-CLL (12) & 5-14 & 7-18 & 0.1-1.1 (0.5%)† \\
AMML (5) & 2-7 & 11-76 & 0.2-0.5 \\
B lymphoma (18) & 33-78 & 2-8 & 0.1-1.0 (0.7%) \\
CML (3) & 1-4 & 2-7 & 0.1-0.8 \\
C-ALL (4) & 1-83 & 0-4 & 0.3-0.7 \\
T-ALL (1) & 1 & 1 & 0.1 \\
ITP† (2) & 8-14 & 8-19 & 0.8-1.2 \\
AHAI† (2) & 5-11 & 6-13 & 0.9-1.0 \\
HCL (18) & 1-82 & 2-92 & 0.1-83 \\
\hline
\end{tabular}
\caption{Analysis on Non-HCL Samples}
\end{table}

\textbf{Immune thrombocytopenia.}
*Range of percentage of positive cells in cell suspensions from peripheral blood.
†Autoimmune hemolytic anaemia; †Percentage of positive cells (mean).

microscope, both double-marker–positive cells (with a tendency for forming aggregates) and scattered cells expressing only one of the antigens could be clearly identified. Moreover, as is shown in Fig 1, the majority of HC spleen suspensions were SHCL-3/B1-positive, and in EPICS C flow cytometer double-marker–positive cells could easily be demonstrated. As compared with the microscope reading, the population of SHCL-3+/B1 – cells was higher, and the proportion of double-marker–positive cells was somewhat lower. The reason for this discrepancy is undoubtedly the aggregation of HCs by B1, which could already be observed in the microscope after incubation with anti-B1 antibody. Therefore, FCM automatically excludes larger cell aggregates from analysis, our assay slightly overestimates the number of SHCL-3+/B1– cells and slightly underestimates (100% v 92%) double-marker–positive cells.

A series of experiments was performed to validate this assay. Thus, negative controls including relevant second-layer antibodies only and first-layer Mabs of similar isotype with no known specificity all gave <1% reactivity. Because B1 and SHCL-3 are expressed in a number of malignant blood diseases, such specimens represented particularly well-suited controls. Thus, as is shown in Table 3, cells from five patients with acute myelomonocytic leukemia (AMML), and from 12 with chronic lymphocytic leukemia (CLL) that expressed one of the antigens were negative (Fig 2A through C). Moreover, cells from 18 patients with B1 + leukemic non-Hodgkin’s lymphoma were also analyzed and likewise showed few (0.1% to 1.0%) double-marker–positive cells. From these data, we conclude that this method unequivocally identifies HCs with no demonstrable positivity on other malignant blood cells.

In an attempt to evaluate the sensitivity of the assay, we added homogenous HCs from spleen tissue (>95% pure) to the T-ALL cell line MOLT-4 and to peripheral blood MNCs. Another admixture experiment using peripheral blood HCs from a HCL patient (49% SHCL-3/B1 positive) was performed to detect differences between the detection of HCs from spleen tissue and HCs from peripheral blood. The results shown in Table 4 and Fig 3 show that double-marker–positive cells in numbers very close to those expected were observed. Such an admixture experiment can, however, only represent an idealized situation in which double-marker–positive cells are identified in a known population of negative cells. To evaluate the assay in HCL patients with possible residual disease, we investigated a larger group of patients in whom double-marker–positive cells were present in percentages varying from 0.5% to 5%. From a patient with 1.2% SHCL-3/B1 positive cells in the peripheral blood MNC suspension (in which morphology did not reveal any HCs), we purified the double-marker–positive cells using FACS on the FCM. As shown in Fig 4, sorted cells (when reanalyzed) were 94% double-marker–positive and on Giemsa-stained smears had the morphology of HCs. Together, the data in Figs 3 and 4 show that the SHCL-3/B1 assay is capable of identifying very small numbers of circulating HCs when morphology and cytochemistry have failed.

When the number of SHCL-3/B1-positive cells was compared with TRAP staining in 18 samples from nine patients, a surprising heterogeneity of reactivity patterns was observed (Fig 5). Thus, although some of the samples showed a close correlation between the double-marker assay and TRAP, two patients were 60% to 82% SHCL-3/B1 positive but TRAP negative. When evaluated on Giemsa-stained smears, one of the patients had >60% circulating HCs, the other patient showed only abnormal MNCs with some indications of HCs. Diagnosis was made on bone core biopsy. Thus, these patients demonstrate the value of the immunologic assay when TRAP staining is negative or when the HC morphology is atypical.

To elucidate the clinical value of this assay further, we
tested ten HCL patients receiving treatment with α-interferon (α-IFN) (2 × 10^6 U/m² three times a week) longitudinally for 3 to 9 months. Figure 6 shows that most patients exhibited a gradual decline in the circulating number of HCs after an unexplained rise on treatment day 1. This decrease in double-marker-positive cells correlated closely with a favorable clinical response to IFN treatment (B. Nielsen, manuscript in preparation). From these data, we conclude that the SHCL-3/B1 assay can be valuable in evaluating treatment response of patients with HCL.

**DISCUSSION**

The diagnosis of HCL is usually based on the demonstration of HCs—often in a spleen specimen—with the well-known morphology and TRAP positivity, but alternatively the diagnosis can be made by bone marrow core biopsy even in the absence of circulating HCs. In addition, cells positive for B cell antigens with a predominantly mature immunologic phenotype are considered strong supportive evidence. No single surface antigen has, however, been found specific for HCs. That HCs express myelomonocytic antigens (Tables 1 and 2) distinguishes this disorder from any other malignant blood disease of B cell origin. Thus, very few, if any, pre-B-ALL, B-CLL or B lymphoma cells express antigens on the Mo- or My- series, but alone the SHCL-3 antigen that, apart from HCL, is expressed exclusively on normal and malignant myeloid cells. Some controversy has existed concerning the reactivity of HCs with myeloid Mabs, especially the Mo1(CD11b), but the data shown in Table 2 clearly show that CD11b antigen reactivity on HCs varies to a large extent from patient to patient. The presence of these myelomonocytic antigens is puzzling, especially in light of the recent demonstration of immunoglobulin gene rearrangements⁵ and the secretion of monoclonal immunoglobulin in HCs. HCs, however, are capable of phagocytosis and lysozyme discharge, as evaluated by electron microscopy⁶ and, in a recent study, we showed functional phagocytic characteristics of HCs. Although the B2 antigen contains both the C3d receptor as well as the receptor for Epstein-Barr virus (EBV), no biologic function has been attributed to the other B cell-related antigens used in this study. The presence of the B1, B2, and B4 antigens on HCs demonstrated here and the reactivity with the plasma cell-associated PCA-1 antigen clearly indicate that the maturational stage of HCs lies between the antigen-activated B lymphoblast and the pre-plasma cell.

In this report, we have shown that the concomitant reactivity of two monoclonal antibodies directed against antigens on different hematopoietic cell lineages enables identification of HCs in cell suspensions from peripheral blood, spleen tissue, and bone marrow. The assay is, however, at a disadvantage in the rare HCL cases without circulating HCs and in those in which suitable cell suspensions from the bone marrow can not be obtained. The double-marker assay was optimized by choosing antibodies that had shown the most consistent reactivity and noticeable high density on HCs and by using admixture experiments and FACS to verify the capacity of
the assay to detect small numbers of HCs. The SHCL-3/B1 test is valuable since HCL can often present with very few symptoms and few circulating HCs. Furthermore, the TRAP test that has been used extensively as a marker of HCL in both clinical and experimental situations is not totally specific for HCs and TRAP-negative HCL cases have been described, and the SHCL-3/B1 assay seems to identify such a TRAP-negative subset of HCs (Fig 5). On the other hand, in three TRAP-positive cases, fewer SHCL-3/B1-positive cells were observed (Fig 5). This can easily be explained, however, by the already mentioned underestimation of double-marker-positive cells. Indeed, microscopic evaluation revealed a much closer correlation to TRAP reactivity in these cases (data not shown).

Attempts to raise Mabs against HCs have yielded antibodies with selective reactivity against either myelomonocytic cells or B cells and no single Mab can yet be claimed to identify HCs.9,21-24 The value of this double-marker test should be evaluated in light of these findings. Choosing malignant blood diseases known to be positive to either SHCL-3 or B1, we demonstrated that only insignificant numbers of double-marker-positive cells can be found in such situations, and analysis of nonmalignant samples has demonstrated minute but consistent numbers of positive cells (Table 3). Present studies in our laboratory are aimed at the characterization and purification of this small nonmalignant counterpart of the HCs since the data given in Fig 4 have shown that this test is powerful enough to enable sorting of HCs even when they constitute as little as 1% of a cell suspension. The number of SHCL-3/B1-positive cells in our study (mean 0.5%) in 25 normal persons differs only slightly from the number of SIgG + cells found in peripheral blood by Machii and Kitani (0.9%)25 and these SHCL-3/B1-positive cells are likely to be the same population as the SIgG + cells.

Figure 5 clearly indicates that TRAP-negative cases of HCL might be disclosed by use of SHCL-3/B1 analysis, thus...
contributing significantly to the diagnostic procedures in HCL. Based on the longitudinal clinical testing performed in HCL patients receiving IFN (Fig 6) we conclude that this assay is valuable both in diagnosis and monitoring of HCL patients. The assay might be envisaged to be of special value in patients treated with Pentostatin (2'-deoxycoformycin), of whom >50% are believed to enter complete remission.6 Finally, we emphasize that since the FITC and PE dyes used here are identifiable in the fluorescence microscope, this double-marker assay can also be used in laboratories without access to flow cytometers.

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A two-color flow cytometry assay for detection of hairy cells using monoclonal antibodies

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