The Diagnosis of Hairy Cell Leukemia Can Be Established By Flow Cytometric Analysis of Peripheral Blood, Even in Patients With Low Levels of Circulating Malignant Cells.

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The Diagnosis of Hairy Cell Leukemia Can Be Established by Flow Cytometric Analysis of Peripheral Blood, Even in Patients With Low Levels of Circulating Malignant Cells

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The diagnosis of hairy cell leukemia (HCL) has traditionally been based on microscopic means. Immunophenotypic analysis of peripheral blood by flow cytometry is not widely recognized as a method for diagnosing HCL, perhaps due to the expectation of low yield of neoplastic cells in patients who are characteristically leukopenic. The abnormal co-expression of CD103, CD25, and intense CD11c and CD20 on monotypic, slightly large B-lymphocytes has previously been shown to be highly characteristic of HCL. We wished to determine if this pattern was valuable in the diagnosis of HCL in leukopenic patients with low levels of neoplastic cells in the peripheral blood. The abnormal immunophenotype above was observed in 25 peripheral blood specimens from patients with unexplained cytopenias or suspected lymphoproliferative processes. Ten of the 25 blood samples exhibited this abnormal phenotype in less than 5% of circulating leukocytes (ranging from <1% to 4%). All 10 patients had other manifestations of HCL, including cytopenias (mean white blood cell count, 1.8 × 10^3/mm^3; hemoglobin, 11.0 gm/dl; platelets, 74 × 10^3/mm^3), splenomegaly, and typical bone marrow morphologic changes. Eight of the 10 patients achieved an excellent response to one course of 2-CDA, with significant improvement of cytopenias (mean white blood cell count: 5.3 × 10^3/mm^3; hemoglobin: 14.4 g/dl; platelets: 181 × 10^3/mm^3) and regression of splenomegaly. One patient had a partial response to alpha interferon and a subsequent complete response to 2-CDA, and one died during treatment. In conclusion, flow cytometric immunophenotyping of peripheral blood is capable of detecting low levels of circulating malignant cells in HCL, even in leukopenic patients. As such, it can be a very useful, non-invasive tool in the diagnosis of this disorder. Am. J. Hematol. 67:223–226, 2001.

Key words: flow cytometry; hairy cell leukemia; diagnosis

INTRODUCTION

Hairy cell leukemia (HCL) is a disorder of B-lymphocytes that typically involves the bone marrow, peripheral blood, and spleen at the time of presentation. The diagnosis of HCL has traditionally been based on microscopic evaluation of the bone marrow and peripheral blood, with confirmatory evidence provided by the presence of tartrate-resistant acid phosphatase (TRAP) in circulating hairy cells [1]. The constellation of splenomegaly, varying degrees of cytopenias, circulating hairy lymphocytes which are TRAP positive, and a bone marrow biopsy showing characteristic morphologic changes [2] poses little difficulty in diagnosis. However, in the peripheral blood, low levels of circulating hairy cells may make their identification by routine light microscopy extremely difficult and may yield ambiguous results.

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A characteristic phenotype of HCL has been demonstrated by flow cytometry (FCM) [3–7]. In the present study we identified cytopenic patients with very low levels of circulating HCL as determined by FCM using an appropriate panel of markers and reviewed their response to HCL-directed chemotherapy.

MATERIALS AND METHODS

Selection of Patients

Between June 1994 and June 1998, 2,012 samples of bone marrow and 687 samples of peripheral blood were referred to the FCM laboratory of Shands Hospital at the University of Florida College of Medicine for evaluation of patients showing unexplained cytopenias or suspected lymphoproliferative disorders. The typical HCL immunophenotype described in Results was demonstrated in 48 patients: 23 samples of bone marrow and 25 samples of peripheral blood. Ten of the 25 samples that showed less than 5% of circulating cells with the phenotype described in this study were selected for clinical and laboratory review. These samples were from 7 males (ages 37–56) and 3 females (ages 48–70). Hairy cells were not identified with certainty microscopically on peripheral blood smears in these patients, and none of the patients were previously treated.

Sample Preparation and Staining

Peripheral blood was collected into EDTA-containing vacuum tubes. Samples were exposed to a 0.15 M NH_4Cl solution to lyse red blood cells, and the remaining cells were washed twice in a phosphate-buffered saline solution containing 0.1% NaN_3 (PBS). Cells were resuspended in cell medium (RPMI) containing antibiotics.

Immunofluorescence Staining and Cell Analysis

Cells were stained as previously described [8,9]. In short, approximately 10^5 cells were exposed to a saturating dilution of fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, or peridinin chlorophyll protein (PerCP)-conjugated monoclonal antibody or nonrelevant, isotype-matched immunoglobulin controls in 96-well microtiter plates (Becton-Dickinson, Labware, Lincoln Park, NJ) on ice for 15 min in the dark. Before analysis, cells in the wells were washed twice with 100 μl of phosphate-buffered saline. The antibodies selected for this study were directed against the following antigens: CD20 (Leu-16, Becton Dickinson Biosciences, BDB, San Jose, CA), CD19 (Leu-12, BDB), CD11c (Leu-M5, BDB), kappa immunoglobulin (Caltag, Burlingame, CA), lambda immunoglobulin (Caltag), kappa immunoglobulin (Biosource, Camarillo, CA), lambda immunoglobulin (Biosource), CD103 (IQ Products, Groningen, The Netherlands), and CD25 (Anti-IL2R, BDB). Two- or three-color FCM was performed by FACScan or FACScalibur (BDB) cytometers. Fluorescence signals were collected using four-decade logarithmic amplification.

Data Analysis

Generally (1–2) × 10^4 total events were analyzed, both without an initial selection (gating) and by "live gating" on mononuclear cells only. In most cases, a subsequent live-gated analysis of CD20-expressing cells only was performed to better determine the phenotype of the B-cells. LYSYS or CellQuest software (BDB) was used for data analysis. The expression of antigens was determined by visual inspection of the graphs as previously described [10] and specified by consensus recommendations on flow cytometric data analysis and interpretation of hematologic neoplasia [11].

RESULTS

The samples from ten patients demonstrated the typical HCL phenotype on <5% of circulating leukocytes. All cases exhibited similar phenotypic findings as shown in Figure 1 which illustrates a representative case. The percentage of abnormal cells detected ranged from less than 1% (5 cases) to 4%. The hairy cells were larger than normal lymphocytes (Fig. 1A) and expressed CD19 (not shown), CD20 (bright) (Fig. 1A–F), CD103 (Fig. 1B), CD25 (Fig. 1C), CD11C (bright) (Fig. 1D), and light chain-restricted surface immunoglobulins (Fig. 1E, F). Non-neoplastic B-cells were often present and tended to obscure the detection of the small population of HCL. In these cases, the hairy cells could be detected more clearly by the analysis of a large number of B-cells (as shown in the "live-gated" graphs E and F in Fig. 1). The normal B-cells were clearly identified by their characteristic bimodal distribution of immunoglobulin light chains, while the hairy cells showed a typical monoclonal pattern (Fig. 1E, F). The separation of abnormal from normal B-cells was facilitated by the use of antibody to CD20, which clearly discriminated the two subpopulations. CD19 expression, on the other hand, was similar on all B-cells and did not help delineate the hairy cells. In fact, the higher CD20 expression of hairy cells helped demonstrate the larger size of these cells and the characteristic coexpression of other antigens in the bivariate displays (Fig. 1). Another finding that we observed in this study (and in other cases of HCL) is that the abnormal cells appeared to nonspecifically bind serum immunoglobulins (probably via their avid IgG Fc receptors) [3,12], resulting in an increase in surface immunoglobulin background fluorescence (Fig. 1E, F).

Table I lists the clinical and laboratory data in the patients studied. All 10 patients had other clinical and/or laboratory evidence of HCL, including varying combinations of peripheral cytopenias, splenomegaly, and cellular bone marrow infiltrate. In further support of the

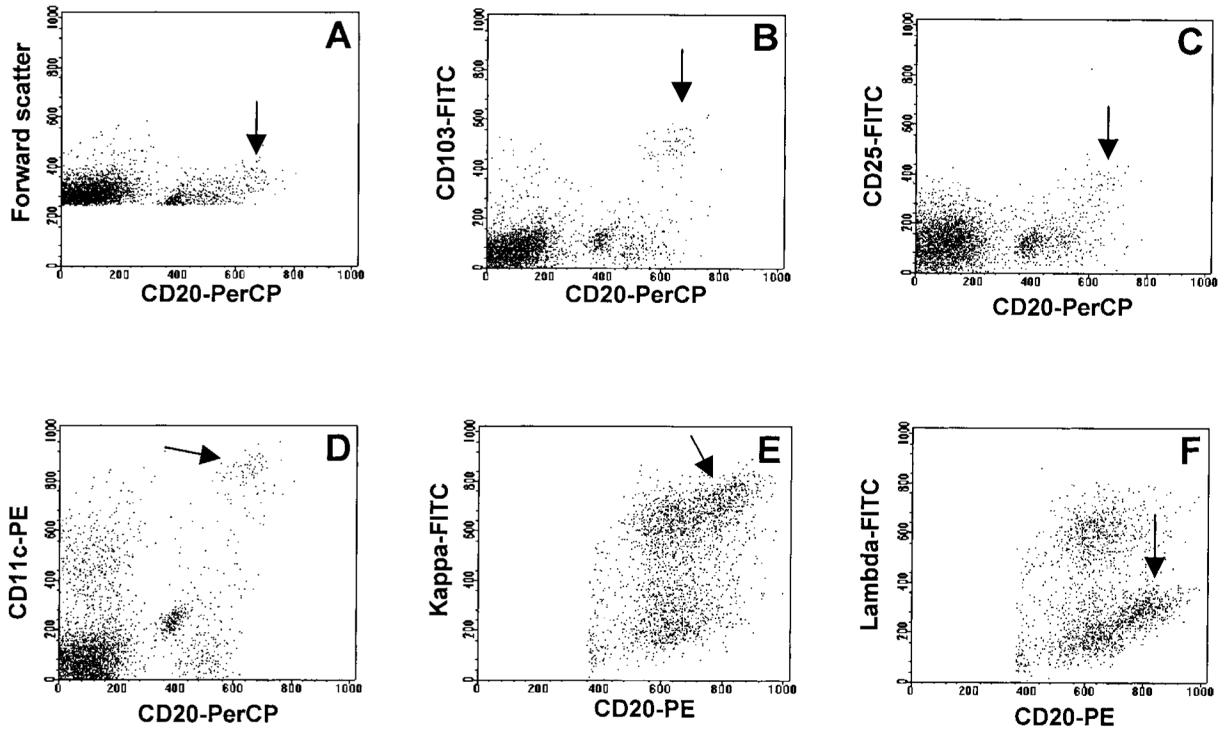

Fig. 1. Flow cytometric analysis of blood in a patient with low levels of circulating hairy cells. Only data from mononuclear cells are displayed (granulocytes have been excluded from the analysis on the basis of their light scatter properties). Hairy cells are recognized by their characteristic phenotypic pattern (arrows). They are slightly larger than normal lymphocytes by forward light scatter (A), express bright CD20 (A–F), and coexpress CD103 (B), CD25 (C), and bright CD11c (D), all recognized by the bright CD20 expression that serves as the common denominator for the identification of hairy cells. Hairy cells in this case are also restricted in the expression of kappa immunoglobulin light chain, whereas less bright CD20-expressing cells are polyclonal B-cells, as demonstrated in E and F. In these latter graphs, a "live gate" is used on CD20-positive cells to analyze a larger number of B-cells than in the other graphs in order to better demonstrate immunoglobulin light chain expression. While the bright CD20 cells express mainly kappa light chains, the less bright CD20 populations consist of a mixture of kappa- and lambda-expressing cells. In both anti-kappa and anti-lambda plots, the hairy cells demonstrate higher fluorescence than their positive and negative polyclonal B-cell counterparts, most likely due to nonspecific binding of serum immunoglobulins.

TABLE I. Effect of Therapy in 10 Patients With <5% Circulating Hairy Cells

	Pre-treatment	Post-treatment (2-CDA)[a]
Range of circulating hairy cells (% of leukocytes)	<1–4	Not analyzed
Mean hemoglobin (g/dl)	11.0	14.4
Mean white blood cell count ($\times 10^3/mm^3$)	1.8	5.3
Mean platelet count ($\times 10^3/mm^3$)	74	181
Splenomegaly	6/10	0/9

[a]One patient achieved a partial response to α-interferon and a subsequent complete response to 2-CDA; one patient expired during treatment with 2-CDA.

diagnosis of HCL, 8 of the 10 patients achieved an excellent clinical response with significant improvement of cytopenias and regression of splenomegaly after one course of 2-chlorodeoxyadenosine (2-CDA). One patient had a partial response to alpha-interferon and a subsequent complete response to 2-CDA, and one patient died during attempted remission induction with 2-CDA.

DISCUSSION

Although traditional morphologic evaluation of the peripheral blood smear is an extremely valuable tool in screening for HCL, the disease may go undetected when very low levels of hairy cells are present. In these cases, even the TRAP stain, widely used to confirm a suspicion of HCL, may not be contributory. Moreover, bone marrow biopsy may sometimes yield material that is inadequate or not sufficiently specific for the diagnosis of HCL. Our experience with the application of FCM to the immunophenotypic diagnosis of HCL demonstrates that this disease can be detected when present in very small amounts in the peripheral blood. This finding was noted by Robbins et al. [6], who studied 161 cases of HCL by

two-color immunofluorescence FCM. Although their analysis focused on the 133 patients with greater than 2% malignant cells in the peripheral blood, circulating hairy cells could be detected even when they represented less than 1% of peripheral blood lymphocytes. In our own study, 10 of 25 patients (40%) whose blood was analyzed by FCM for diagnostic purposes and found to contain HCL showed a very small number of circulating malignant cells (from <1% to 4%). Despite its presence in very low amounts, this population was readily identifiable by carefully examining the graphic phenotypic patterns, as advocated in a recent FCM consensus conference [11], rather than by relying on percentage of cells positive for each antibody used. In our series, the diagnosis of HCL was further supported by the rapid clinical improvement following one course of 2-CDA therapy in all but one patient, who died during treatment.

The value of FCM analysis of peripheral blood in the diagnosis of HCL appears to be under-recognized by many physicians, perhaps relating to a lack of familiarity with the capabilities of this technique and a misperception that leukopenic patients have too few circulating hairy cells for detection. However, as a rapid and non-invasive tool, it is ideally suited to the diagnosis and therapeutic monitoring of diseases like HCL, which are characterized by the presence of low numbers of circulating malignant cells. These benefits of FCM raise the issue of whether it can supplant more invasive procedures like bone marrow biopsy or splenectomy in the diagnosis of HCL. At the present time, the false-negative rate of FCM as a diagnostic modality in HCL is unknown, so that a negative result by FCM cannot equate with absence of disease. Also, we did not attempt to exhaustively analyze our database for false-positive cases. However, a characteristic phenotypic pattern based on an appropriate antibody combination appears to be highly specific for HCL. Our data supports the view that FCM can be helpful in any patient suspected of having HCL, even if significant leukopenia is present. Although a negative result would not necessarily rule out HCL, positive findings would be highly supportive of the diagnosis and might obviate further diagnostic measures.

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