Flow Cytometry in Hairy Cell Leukemia Before and During Interferon Alfa-2b Therapy

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Mononuclear cells from 15 patients with hairy cell leukemia were studied before and during therapy with interferon alfa-2b (IFN) by regular peripheral blood differential counts and flow cytometry, using a panel of monoclonal antibodies (Moab). Seven leukemic phase patients (Group 1) had a mean leukocyte count of 48,870/μl at entry with a mean absolute hairy cell (HC) count of 40,100/μl. After 3 months on IFN, both parameters decreased significantly (WBC 3,500/μl; HC count 130/μl). In eight patients with a cytopenic form of the disease (Group 2) the mean leukocyte count rose from 2950/μl to 3890/μl while the mean absolute HC count decreased from 300/μl to 120/μl. The morphologic shifts correlated well with changes in the Moab reaction pattern. In Group 1 the activity of all Moab decreased significantly. In Group 2, only cells expressing Leu 3a and Leu 11a (a marker of natural killer cells) showed a significant shift, the latter increasing from 170/μl to 360/μl. This increase in natural killer cell antigen expression was not obvious based on routine morphologic observations alone. We show that flow cytometry may be a useful adjunct in monitoring the response of HCL to therapy. Changes in populations of cells that may be difficult to discriminate on morphologic grounds alone may be observed.

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STRIKING RESULTS have been published on the efficacy of interferon in the treatment of hairy cell leukemia (HCL).[1-4] Of 64 patients treated with interferon alfa-2b (IFN) 5% had a complete response with apparent eradication of hairy cells from the bone marrow, 70% had normalization of all three peripheral blood cell counts and decreased involvement in the bone marrow, and 14% showed improvement in at least one blood count.[3] Patients in the leukemic phase of the disease in particular have been observed to show a rapid decline in the number of hairy cells in the peripheral circulation in response to interferon. The mechanism of action of interferon in patients with HCL has not yet been defined. It has been demonstrated to directly inhibit the proliferation of various leukemic cell lines *in vitro*[5] and to induce differentiation, *eg*, in promyelocytic leukemic cells.[6] An indirect mechanism of action through the activity of natural killer (NK) cells has been implied, in that NK-cell activity has been shown to be severely depressed in HCL[7] and to increase in patients with interferon therapy.[4]

Although the issue was debated for a long time, the hairy cell is thought to be of the B-lymphocyte lineage in nearly all patients. The neoplastic cells show clonal immunoglobulin gene rearrangements and react with monoclonal antibodies (Moab) directed against B-cell antigens.[8-11] We report 15 patients with HCL (seven in the leukemic phase of the disease) who were studied with a panel of Moab directed against B-lymphocytes, T-lymphocytes and their subsets, monocytes, and NK-cells by flow cytometry (FC) prior to initiation of interferon therapy, and at three monthly intervals while on therapy. Thus, we were able to follow the shift in Moab reactivity and compare it to the morphologic changes observed in the peripheral blood differential count as a result of interferon therapy.

Materials and Methods

The diagnosis of HCL was based on the typical morphologic appearance of the leukemic cells in the peripheral blood and in the bone marrow[12] as well as positive staining with tartrate-resistant acid phosphatase.[13] All patients had significant anemia (hemoglobin < 10 g/dl or need for transfusion), thrombocytopenia (platelets < 100,000/μl), neutropenia (neutrophils < 1000 cells/μl) and/or leukemia

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TABLE 1. Panel of Monoclonal Antibodies Used for FC

Monoclonal antibody	Specificity	Composition	Reference no.
Anti-Leu 1	Human T-lymphocyte	Mouse Ig G2a	15
Anti-Leu 2a	Human T-lymphocyte (cytoxic/suppressor)	Mouse Ig G1	16
Anti-Leu 3a	Human T-lymphocyte (helper/inducer)	Mouse Ig G1	17
Anti-HLA-DR	B-lymphocyte, monocyte, activated T-cell	Mouse Ig G2a	18
Anti-B1	B-lymphocyte	Mouse Ig G2a	19
Anti-BA1	B-lymphocyte	Mouse Ig M	20
Anti-Leu 11a	Large granular lymphocyte, NK cell	Mouse Ig G1	21
Anti-Leu M1	Granulocyte, monocyte	Mouse Ig G2b	22
Anti-Leu M3	Monocyte	Mouse Ig G2b	23
Anti-kappa	Human immunoglobulins with kappa light chains	Mouse Ig G1	24
Anti-lambda	Human immunoglobulins with lambda light chains	Mouse Ig G1	25

FC: flow cytometry.

(leukocyte count > 10,000 cells/μl with more than 50% hairy cells). All had failed prior splenectomy. Some had failed additional chemotherapy. Therapy with IFN was given at a dose of 2×10^6 U/m^2 body surface area. Details on the clinical aspects of this study have been reported on previously.[3] FC was performed prior to initiating IFN therapy and at 3 monthly intervals thereafter, while patients continued on IFN. On the day of FC, a complete blood count and a peripheral blood differential count were performed as well.

Isolation of Mononuclear Cells

For FC, peripheral blood was collected in ethylene diamine tetraacetic acid (EDTA). Mononuclear cells were isolated by Ficoll Hypaque centrifugation as previously described.[14]

Flow Cytometry

After incubation in serum-free media for 1 hour at 37°C, mononuclear cells were reacted for 20 minutes with each of a panel of antibodies. Some antibodies were conjugated to fluorescein or phycoerythrin. Other antibodies required a second-step reagent, which was either fluorescein-conjugated goat anti-mouse IgG, or rat anti-mouse IgM. Irrelevant antibodies of the same subclass were used as controls. For each antibody 10,000 ungated mononuclear cells were studied on a FACS analyzer (Becton Dickinson, Mountainview, CA). Cells that showed fluorescence intensity exceeding that of the cells stained with irrelevant antibody were considered positive and were expressed as percentages.

Panel of Moabs

To immunologically identify subsets of mononuclear cells, a panel of 11 Moab was used (Table 1). This allowed for identification of T-lymphocytes and their "helper" (Class II major histocompatibility antigen recognizing) and "suppressor" (Class I major histocompatibility antigen recognizing) subsets, B-lymphocytes, NK cells, and monocytes. In a few instances, not enough cells were available for testing of all 11 Moab. In these cases, T- and B-lymphocyte antigens were tested for preferentially.

Statistical Analysis

For statistical analysis the peripheral blood differential count was expressed as absolute number of cells per microliter (percentage of cell types on morphologic differential count times absolute leukocyte count per microliter). Thus, the dramatic changes in total count as seen especially in the leukemic patients treated with IFN could be taken into account. Lymphocytes, monocytes, and hairy cells were counted as mononuclear cells. Moab reactivity was expressed as absolute number of mononuclear cells per microliter reacting with a given Moab (percentage of cells reacting with a Moab times percent of mononuclear cells on differential count times leukocyte count per microliter). Thereby, we assumed the cellular composition of the mononuclear cell suspension isolated by density gradient centrifugation to be representative of the patient's mononuclear cell population. We also assumed that therapy did not alter the recovery of mononuclear cells.

Patients were divided into two groups based on their peripheral leukocyte count. The predominance of hairy cells in the peripheral blood and the mononuclear cell preparation of the seven leukemic patients (leukocyte count $\geq$ 10,000/μl with >50% hairy cells) allowed for designation of these patients as Group 1. The 8 nonleukemic patients whose peripheral blood was not dominated by a single cell type, comprised Group 2. The two groups were compared regarding the initial results and subsequent changes after 3 months of therapy with IFN. The paired t-test was applied to determine the significance of a shift of mean values from time 0 (time of starting IFN) to 3 months on therapy within each of the two study groups.

Results

A total of 15 patients were studied; seven were in the leukemic phase of the disease, while eight patients suffered from varying types and degrees of cytopenias as required for entry to the clinical trial.[3]

Changes in Peripheral Blood Counts

In Group 1, the leukocyte count at entry was 48,870 $\pm$ 35,390/μl, with an absolute hairy cell count of 40,100

TABLE 2. Change in Peripheral Blood* Leukocyte Count and Absolute Differential Count†

	Group 1			Group 2		
	Month			Month		
Cell type	0	3	*P*	0	3	*P*
Leukocytes/μl	48870 ± 35290	3500 ± 1050	0.01	2950 ± 1260	3890 ± 1160	0.19
Granulocytes/μl	970 ± 790	1220 ± 660	0.55	780 ± 490	1880 ± 1400	0.05
Lymphocytes/μl	7320 ± 5290	1920 ± 1170	0.02	1710 ± 870	1620 ± 920	0.55
Monocytes/μl	420 ± 530	180 ± 220	0.38	20 ± 30	270 ± 220	0.03
Hairy cells/μl	40100 ± 29740	130 ± 190	0.01	300 ± 330	120 ± 220	0.2

* The peripheral blood differential count was expressed as absolute number of cells/μl (percent of cell type on differential count times absolute leukocytes/μl). Group 1 consists of patients with leukemic phase hairy cell leukemia (leukocytes ≥ 10,000/μl), Group 2 of patients with a cytopenic form of the disease. The significance of changes in values from time 0 to 3 months was examined by the paired t-test as listed under *P*.

† Mean ± SD.

± 29,740/μl (Table 2). After 3 months of IFN therapy, the leukocyte count had decreased significantly to 3500 ± 1050/μl, and the absolute hairy cell count to 130 ± 190/μl (two patients had complete morphologic disappearance of hairy cells from the peripheral blood). During the same interval the lymphocyte count decreased significantly, but absolute granulocyte or monocyte counts did not increase significantly. In the succeeding 3-month observation periods, no significant further changes were seen except for a continuous increase in granulocytes.

Group 2 consisted of eight patients with a low or normal leukocyte count at entry (2950 ± 1260/μl) and a relatively low absolute hairy cell count (300 ± 300/μl). After 3 months of IFN therapy, the leukocyte count rose to 3890 ± 1160/μl while the absolute hairy cell count decreased to 120 ± 220/μl (in this group, three patients had complete clearance of circulating hairy cells). Granulocytes and monocytes increased while lymphocytes showed no significant shift. The changes in absolute granulocyte and monocyte count are statistically significant. In the succeeding 3-month observation periods no significant changes were seen; only four of eight patients, however, were available for analysis at those times.

Changes in Mononuclear Surface Antigen Expression

Generally the changes in peripheral blood morphology correlated well with changes in the reaction pattern with the Moab tested (Table 3, granulocytes are not included in the Ficoll layer of mononuclear cells and therefore were not assessed). In Group 1 there was a significant decrease of all antigens tested, when expressed as absolute number of mononuclear cells reacting with a Moab. This was true for the T-cell markers Leu 1, Leu 2a, and Leu 3a. All B-cell markers, monocyte/granulocyte markers, and the Leu-11a antigen also showed a significant decrease in this group. The "helper/suppressor" cell ratio, calculated by dividing the mean Leu 3a values by the mean Leu 2a values of a whole group at a given time, did not change

TABLE 3. Change in Antigen Expression* of Mononuclear Cells†

	Group 1			Group 2		
	Month			Month		
Monoclonal antibody	0	3	*P*	0	3	*P*
Leu 1	7130 ± 4540	1380 ± 1120	0.01	890 ± 470	1450 ± 510	0.1
Leu 2a	2660 ± 1160	790 ± 730	0.002	410 ± 130	610 ± 320	0.2
Leu 3a	2510 ± 910	740 ± 570	0.001	640 ± 240	910 ± 230	0.01
HLA-DR	34330 ± 34380	250 ± 190	0.04	350 ± 380	580 ± 690	0.6
B1	37810 ± 29650	440 ± 270	0.04	480 ± 230	390 ± 160	0.6
BA1	43850 ± 33750	250 ± 120	0.02	420 ± 270	230 ± 170	0.1
Kappa	29160 ± 17910	260 ± 200	0.01	220 ± 310	270 ± 70	0.9
Lambda	10450 ± 6500	210 ± 130	0.01	110 ± 140	140 ± 90	0.4
Leu 11a	1890 ± 1260	250 ± 160	0.01	170 ± 150	360 ± 150	0.002
Leu M1	2230 ± 2050	240 ± 170	0.05	100 ± 160	140 ± 80	0.6
Leu M3	1690 ± 1130	200 ± 150	0.009	80 ± 130	270 ± 100	0.06

* Antigen expression was assessed by absolute number of mononuclear cells/μl reacting with a given monoclonal antibody (percentage of cells reacting with a monoclonal antibody times percentage of mononuclear cells on differential count times leukocytes/μl). Groups and *P* value as in Table 2.

† Mean ± SD.

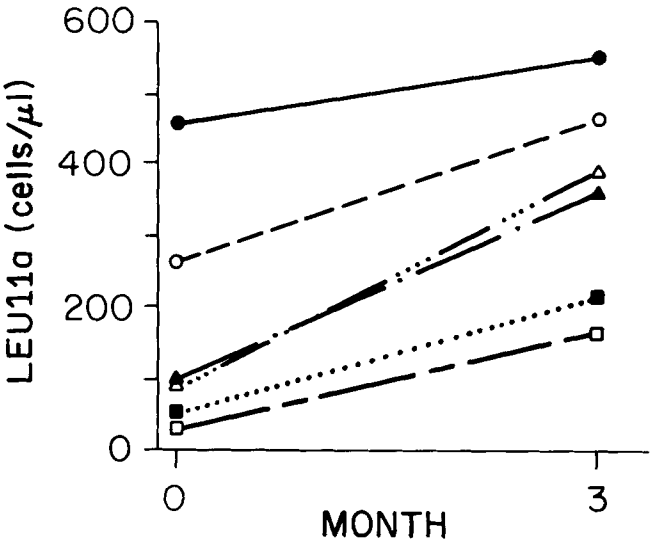

FIG. 1. Change in Leu-11a expression (number of mononuclear cells/μl reacting with Anti Leu-11a) for each individual patient tested in Group 2 initially and after 3 months of IFN therapy. An increase is shown in each instance.

significantly from the time of entry into the study to the subsequent observation periods. In two patients who had a "helper/suppressor" ratio of less than 1 at entry, it did not increase with therapy.

In Group 2, the lack of a significant morphologic shift in the number of lymphocytes and hairy cells was reflected by a lack of a significant shift for most of the Moab tested from time 0 of the study to the 3-month follow-up with two exceptions.

The first exception is Leu-3a, indicating an increase in "helper" T-lymphocytes at 3 months, although, as in Group 1, the ratio of "helper" to "suppressor" lymphocytes did not change significantly, nor did it change in individual patients during IFN therapy in either group. The other exception is Leu-11a, indicating a significant increase of NK-cell antigen expression after 3 months of IFN therapy (Fig. 1). There was no significant change with further therapy, although cells of only three patients were available for analysis at 6 and 9 months of therapy. The "helper/suppressor" ratio for the whole group did not change significantly; two patients had a ratio of less than 1 at diagnosis, which increased to normal in one patient with IFN therapy.

Discussion

Several studies in the more recent literature suggest the hairy cell to be of B-lymphocytic origin.[8-11,26-28] This concept is clearly supported by our studies using FC on a group of leukemic and cytopenic patients with hairy cell leukemia before and during IFN therapy. While both T- and B-cell markers show a significant decrease in Group 1 as hairy cells disappear from the mononuclear cell layer after 3 months of IFN therapy, it is the B-cell antigens (B1, BA1, HLA-DR, K, and λ) that initially react with the great majority of cells tested (mostly hairy cells) and then decrease markedly. T-cell markers (Leu 1, 2a, 3a) decrease as well; however, their baseline values are less pronounced. Group 2, which has only few peripheral hairy cells even initially, shows a low baseline of cells expressing B1, BA1, HLA-DR, K, and λ antigens and no significant change after IFN therapy, although in this group, too, other disease parameters (*e.g.*, granulocytopenia) normalize.

The number of NK cells in the peripheral blood was assessed in this study by determining the presence of the Leu 11a antigen.[21] The results as obtained in Groups 1 and 2 seemingly contradict each other, as Leu-11a expression decreases significantly in Group 1 whereas it increases significantly in Group 2. For Group 1, a significant decrease of all Moab tested is observed with the decrease of hairy cells in the peripheral blood, although none of the Moab used identify antigens specific for hairy cells. This can be explained by a varying fraction of hairy cells cross-reacting with each Moab tested at time 0, then showing decreased activity after 3 months, when peripheral blood hairy cells have decreased. Assuming that a substantial number of hairy cells react nonspecifically with anti Leu-11a initially in Group 1, a decrease in Leu-11a expression is to be expected after three months and therefore does not represent a true decrease in NK cells. In Group 2, however, few circulating hairy cells are present initially and no single cell type clearly dominates the mononuclear cell differential. Therefore, no interference or nonspecific "cross-reactivity" is present, and the demonstration of a significant increase of Leu-11a expression may reflect a true increase in NK-cells in HCL treated with IFN for 3 months. This observation is important, as the shift in NK antigen expression in Group 2 occurs independent of a major morphologic shift in the peripheral blood differential count and, thus, may represent a shift in functional cell subsets. However, our studies reflect a significant shift in NK-cell antigen expression only. They do not assess functional NK activity. Therefore, the observed shift could also be due to an expansion of (rare) non-NK T-lymphocytes that react with Leu-11a.

Using a functional assay, Ozer *et al.*[29] were able to demonstrate an increase in NK activity from subnormal to normal levels in seven patients with HCL undergoing therapy with IFN. Foon *et al.*,[4] using a functional assay, also demonstrated improved NK-cell activity in patients with HCL on interferon therapy, but were unable to show an increase in cells expressing the Leu-11a antigen. Their analysis, however, was based on relative rather than absolute cell counts. Semenzato *et al.*[30] also showed increased NK activity after 3 months of therapy with IFN and a further increase at 6 months in three patients with HCL. Bardawil *et al.*[31] reported that the number of large granular lymphocytes (thought to represent NK cells[32])

increased after 2 months of IFN therapy in 11 of 12 patients with HCL, although their values continued to fluctuate at further observation periods. The number of patients whose cells were available for analysis at those times in this report is too small to allow firm conclusions. Therefore, further studies on HCL that address the question of NK activity prior to IFN therapy and during longer periods of subsequent therapy are needed. Those should use a functional assay with high sensitivity in addition to morphologic and immunologic analysis.

This study shows the utility of FC in a research format in conjunction with morphology to monitor the response of HCL to therapeutic intervention. While these data, generated by FC, generally parallel the changes in the peripheral blood, FC offers several potential advantages over the use of morphologic findings alone. In a routine differential count, a significant degree of interobserver variation can be seen due to the small numbers of cells counted (usually 100 or 200). With FC, however, 10,000 cells are counted with high objectivity, greatly increasing the degree of precision by which minor changes can be observed. By using right-angle light scatter FC, hairy cells can be accurately identified and quantitatively assessed.[33] In addition, FC offers information derived through the ability of Moab to discriminate further subsets of cells that cannot routinely be discriminated on grounds of morphology alone. As more specific Moab become available and multiparameter FC allows for the detection of multiple antigens on individual cells, this will become even more significant. Hopefully, we will then be able not only to monitor changes in cell populations in diseases like HCL but also to define the mechanisms underlying the changes.

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