Flow Cytometric Characterization of Acute Myeloid Leukemia. Part 1. Significance of Light Scattering Properties


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Acute leukemias are classified using the morphological and cytochemical criteria set forward by the French, American and British (FAB) group. Immunophenotyping is helpful for the differential diagnosis but is secondary to the morphological criteria. Immunophenotyping performed by flow cytometry, however, can yield valuable information on cell morphology in addition to cell surface antigen expression. To provide a basis of a combined evaluation of both morphology, i.e. light scattering, and immunophenotype by flow cytometry, we have compared the light scattering profiles of 70 patients newly diagnosed with acute leukemia with normal bone marrow and related the findings to the FAB classification. Three main light scattering profiles were observed in the bone marrow aspirates of the 70 patients: A subpopulation characterized by a predominant cell cluster with low forward and orthogonal light scattering, contained only by all patients diagnosed as acute lymphoblastic leukemia, acute undifferentiated leukemia, and acute non-lymphocytic leukemia M6 and M1. B subpopulation is characterized by a predominant cell cluster with large forward and low to high orthogonal light scattering. Category B contained the majority of patients classified as M2; the M3 leukemias were categorized as C subpopulation characterized by a predominant cell cluster with low forward and high orthogonal light scattering that branches towards regions with larger light scattering. Categories C and D contained the majority of the patients classified as M4. Category C was specific for M4 and M6o leukemias. The patients diagnosed as M4 were heterogeneous and equally distributed over the B and C categories. The clear relationship found between the FAB classification and by the light scattering profile of the acute leukemias enhances the importance of the flow cytometric classification of leukemias. In contrast with light microscopy, flow cytometry can now provide the hematologist with an objective technique to classify leukemias by the simultaneous assessment of cell surface antigen expression and cell morphology, i.e. light scattering.

INTRODUCTION

The classification of acute leukemias can be based on various cellular markers. The most widely accepted and applied classification is based on morphological and cytochemical criteria, proposed by the FAB group in 1975 (1) and revised in 1988 (2). In addition to the morphological evaluation by light microscopy, immunophenotyping has become a powerful tool in characterizing and subdividing acute leukemias (3). Immunophenotyping is most often performed by flow cytometry in which cell surface antigen expression and the light scattering signals of the cells passing the sensing area are measured simultaneously. The information on the cell morphology of the leukemic cells, i.e. light scattering, is often neglected. This is due to the lack of an established correlation between the cell morphology of acute leukemias by light microscopy and by light scattering.

In flow cytometry, forward light scattering is related to cell size (4), and orthogonal light scattering is a measure of cell granularity (5,6). The combination of both light scattering parameters allows a discrimination between neutrophils, eosinophils, basophils, monocytes, granular, and non-granular lymphocytes in normal peripheral blood (6–8). Although in normal bone marrow aspirates the positions of each of these cell populations are identical to those in peripheral blood, the precursors of each of the cell lineages in bone marrow obscure the positions of their end-stages. However, the position of non-lineage committed CD34+ cells is well defined (9,10) and the pathways followed through the corresponding scatter of both light scattering parameters during erythroid, B-lymphoid, and leukoyd lineages are described and are nearly identical in normal individuals (9–14). In this study we have compared the light scattering profile of normal bone marrow cells with bone marrow cells of patients with acute leukemias in order to investigate a potential relationship with the FAB classification and to establish the basis for a combined flow cytometric evaluation of morphology, i.e. light scattering and immunophenotype.

MATERIALS AND METHODS

Cell Preparation

Seventy consecutive adult patients, admitted to the Department of Internal Medicine of the University of Münster, Germany, with newly diagnosed acute leukemia, were included in the study. Bone marrow aspirates were taken from the posterior iliac crest. The diagnosis and classification of the leukemias were based on light microscopy of Pappenheim stained slides, and on cytochemical reaction with PAS, myeloperoxidase, and esterase. All slides were reviewed by two independent cytologists according to the criteria of the FAB classification (2). Bone marrow aspirates of the patients and of more than 50 healthy consenting volunteers were prepared for flow cytometric analysis using NH4Cl lysing. One volume of bone marrow was diluted with 14 volumes of the lysing solution [10 mM EDTA, 0.17 M NH4Cl in H2O (pH 7.3)] and gently mixed. The cells were lysed for 3–5 min and centrifuged at 200 g for 5 min at room temperature. The pellet was resuspended in a volume of RPMI-1640 (Whittaker, Walkersville, MD) 14 times larger than the original bone marrow volume and centrifuged at 200 g for 5 min. This washing step

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Figure 1. Correlative display of forward and transformed orthogonal light scattering of a representative NH4Cl lysed normal bone marrow aspirate. The positions of the neutrophils (N), eosinophils (Eo), monocytes (M), lymphocytes (L), erythrocytes (E), and progenitor cells (X) are indicated. The arrows indicate the route followed during neutrophil development (→), monocyte development (→→→), eosinophil development (→→→), B-lymphocyte development (→→→→), and erythrocyte development (→→→→→).

was repeated twice and the cells were finally resuspended in phosphate-buffered saline (PBS) containing 1% bovine serum albumin, 1% penicillin/streptomycin and 20 mm Hapes (pH 7.3). The cell concentration was adjusted to 1 x 10^7/ml and resuspended in 1 ml of 1% paraformaldehyde in PBS. For instrument standardization 100 μl of cell suspension was immunofluorescently labeled with 20 μl of pre titered CD3 [Anti-Leu4 FITC, Becton Dickinson Immunocytometry Systems (BDIS), San Jose]. After incubating for 20 min on ice, the cells were washed once with 2 ml of the PBS solution at 4°C and resuspended in 1 ml of 1% paraformaldehyde in PBS.

Flow Cytometric Analysis

Flow cytometric analysis was performed on a FacScan (BDIS). The light scattering parameters were standardized by gating on the fluorescence intensity of CD3+ lymphocytes followed by an adjustment of both light scattering detectors to locate the CD3+ lymphocytes in a standard position. Data acquisition and analysis was performed with the FacScan Research Software (BDIS). The orthogonal light scattering signals were transformed according to a polynomial function in order to increase the orthogonal light scattering resolution (15). Forward light scattering and orthogonal light scattering were determined for each cell and stored in listmode data files containing 30,000 cells.

RESULTS

Light Scattering Profile of Normal Human Bone Marrow Aspirates

The light scattering profile of more than 50 normal bone marrow aspirates was studied. Figure 1 shows a typical example of a correlative display of forward and transformed orthogonal light scattering of normal human bone marrow cells. The orthogonal light scattering signals were transformed with a polynomial function in order to emphasize the light scattering region typical for lymphocytes and blast (15). The position of the end maturational stages of each of the cell lineages are indicated in the figure (7,10,15). The position of the non-lineage committed progenitor cells as defined by the expression of the CD34 antigen and the lack of the CD10, CD33, CD38, and CD71 antigens (9) are indicated by X (9,10). The arrows in Figure 1 start at the position of these progenitor cells and follow specific pathways towards the position of the end maturational stages, i.e. erythrocytes, B-lymphocytes, monocytes, eosinophils, and neutrophils. The validation of the position of these arrows was obtained by morphological examination of cells sorted along these maturational pathways (8–14).

Heterogeneity in Light Scattering Profile of Acute Leukemias

The light scattering profile of the bone marrow aspirates of 70 patients with newly diagnosed acute leukemia was analyzed and compared with the normal profile. The most obvious difference with normal bone marrow light scattering profiles is the localization of the majority of cells in one specific light scattering region in contrast to the multiple cell clusters observed in the normal bone marrow aspirates. The position in the correlative display of both the light scattering parameters of the predominant cell cluster varied considerably between the acute leukemias. However, after a careful analysis of these light scattering distribution patterns and a comparison between the patients, three prominent patterns could be distinguished.

The first main pattern (A) is characterized by an accumulation of cells with low forward and orthogonal light scattering properties. The second main pattern (B) is characterized by an accumulation of cells with intermediate to high forward light scattering and low to high orthogonal light scattering. The third pattern (C) is characterized by an accumulation of cells with low forward and orthogonal light scattering but branching towards higher light scattering regions by both forward and orthogonal light scattering. Within each light scattering pattern the position of the cell cluster or the extent of branching differed, which led to subclassifications within each main pattern.

Figure 2 illustrates the typical light scattering profiles of pattern A. Two categories are distinguished. The first, A1, is characterized by an accumulation of cells in a light scattering region with low forward and low orthogonal light scattering signals (Figure 2a); note the presence of cells with larger orthogonal light scattering signals which are non-nucleated erythrocytes and neutrophils and are indicated with E and N, respectively. The second category is A2 in which the cells are more spread out over the dynamic range of the forward light scattering (Figure 2b).

In Figure 3, the typical light scattering profiles of pattern B are illustrated. Three categories are distinguished. B1 is characterized by an accumulation of
cells in a light scattering region typical for normal CD34+ progenitor cells (9,10) (X in Figure 1), i.e., intermediate forward and orthogonal light scattering signals (Figure 3a). In B, the cells are accumulated in a position typical of normal myeloblasts and promyelocytes, i.e., high forward and orthogonal light scattering signals (14) (Figure 3b). In B, the cells are accumulated in a position typical of normal monocytes, i.e., intermediate forward and high orthogonal light scattering signals (10,15) (Figure 3c). In each of these examples the presence of normal lymphocytes can be observed, indicated by L in Figure 3. These cell clusters mainly consist of CD3+ lymphocytes (data not shown).

In contrast to patterns A and B, where the predominant cell cluster is relatively homogeneous, pattern C discloses signs of differentiation towards separate lineages, as indicated by branching of the dominant cell population as illustrated in Figure 3. Four categories are distinguished in pattern C. C_i is characterized by an accumulation of cells in a light scattering region with low orthogonal and intermediate forward light scattering, with a subtle split, where one branch is pointed towards the light scattering region typical for normal monocytes and the other towards the region typical for normal maturing neutrophils (Figure 4a). C is similar to C_i but more than 10% of the cells are accumulated in one of the
branches (Figure 4b). C\textsubscript{1} has a similar scatter profile to C\textsubscript{4}, but the majority of the cells is accumulated in one of the branches (Figure 4c). C\textsubscript{4} is characterized by an accumulation of cells in a light scattering region on top of the normal lymphocytes, which are indicated by L in Figure 4c and d. The acute leukemias with this scatter profile show branching of the bulk population towards regions with higher forward and higher orthogonal light scattering (Figure 4d). A summary of the characteristic light scattering features is presented in Table 1.

**Comparison of Light Scattering Profiles with FAB Classification**

Seventy patients were analyzed. Using FAB criteria three patients were classified as acute undifferentiated leukemia (AUL), seven as acute lymphoblastic leukemia (ALL), and 60 as acute non-lymphocytic leukemia (ANLL), of which seven were classified as M1, 14 as M2, four as M3, 13 as M4, two as M4eo, 14 as M5, four as M6, and two as M7. The light scattering profile of each leukemia could be assigned into one of the categories A\textsubscript{1}, B\textsubscript{1}, C\textsubscript{1}. A comparison between the light scattering classification and the FAB classification is shown in Table 2.

The three bone marrow aspirates classified as undifferentiated leukemia and the seven acute lymphoblastic leukemias were all classified in category A based on light scattering. In addition, all four M6 and five of seven M1 leukemias were clustered in category A. All M2 leukemias had higher light scattering signals (category B and C) and 10 of the 14 M2 bone marrow samples showed branching towards regions typical for more mature cells in the normal bone marrow. This suggests the heterogeneity and maturation of the leukemia cells. The four M3 leukemias were homogeneous and all clustered in the B1 and B2 category. The light scattering profiles of the M4 leukemias were equally distributed over the B and C categories, but no scattering profile fitted into category A. This is indicative of a large heterogeneity within the M4 leukemias. Both bone marrow aspirates classified as M4eo had typical profiles of the C3 category, which indicates a high degree of diversity within the samples. Seven of 14 acute monocytic leukemias (M5) were categorized in B1 and no M5 leukemias were found in category A.
TABLE 2. Comparison of Classification by Light Scattering and Morphology with FAB Criteria.

<table>
<thead>
<tr>
<th>Classification* by FAB</th>
<th>A₁</th>
<th>A₂</th>
<th>B₁</th>
<th>B₂</th>
<th>B₃</th>
<th>C₁</th>
<th>C₂</th>
<th>C₇</th>
<th>C₈</th>
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<td></td>
<td>46</td>
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<td>ANLL</td>
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<td></td>
<td>33, 56, 80, 81</td>
<td></td>
<td>55, 74</td>
<td></td>
<td>10, 76</td>
<td>9, 16, 29, 35, 68, 73, 75</td>
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</tr>
<tr>
<td>M1</td>
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<td>7, 51, 87</td>
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<td>M₇</td>
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*A (AUL) Acute undifferentiated leukemia; (ALL) acute lymphocytic leukemia; (ANLL) acute non-lymphocytic leukemia; (M1-M7) FAB classification.

Classification by light scattering (A₁, A₂) classification by light scattering.

Patients were assigned a number and classified by the FAB and light scattering criteria.

Categorization of the leukemic samples is based on the position of the majority of the cells in the light scattering display. However, additional minor cell populations can frequently be identified, as is illustrated in Figure 5 for a patient classified as M6 by FAB and A₁ by light scattering. The position of these minor subpopulations is often abnormal compared to the scatter profile of normal bone marrow cells as indicated with arrows in Figure 5, suggesting maturational defects. Additional cell populations with abnormal light scattering characteristics were found in 42 of the 70 leukemias (60%).

** DISCUSSION **

The use of light scattering measurements of cells in flow cytometry is often restricted to a discriminatory function for studying the expression of cell surface antigens. In this study we have extended this function to an assessment of cell morphology in patients diagnosed with acute leukemia. The detailed knowledge of the position of the end maturational stages of each cell lineage in the correlation of forward and orthogonal light scattering, as well as the position of their precursors (6-15), allowed a comparison between normal light scattering profiles and those found in the bone marrow aspirates of patients with acute leukemia. Within the leukemic patients three major light scattering patterns were distinguished based on the position of the dominant cell population and the heterogeneity within this cell population (Table 1).

In comparison with the conventional FAB classification, category A included acute lymphoblastic, acute undifferentiated, and the majority of M1 and all M6 leukemias. Morphologically the blasts in these myeloid leukemias lack features of differentiation, which is in concordance with low orthogonal light scattering (lack of granularity). Erythroid precursors present in M6 leukemias and normal bone marrow aspirates also lack granularity as indicated by the low orthogonal light scattering. Although all four M6 leukemias were classified in the A categories by light scattering, small populations of cells were found in these patients with larger orthogonal light scattering indicative for the presence of cells of myeloid origin (Figure 5). The distinction between AUL, ALL, and ANLL M1 and M6 (pattern A₁,₂) from ANLL M2, 3, 4, 5, and 7 (pattern B₁,₂,₃) by light scattering measurements improves the accuracy of leukemia classification as a further discrimination between AUL, ALL, and ANLL M1 and M6 can be achieved by the simultaneous assessment of the antigenic profile, i.e. B- and T-lymphoid lineage specific.
antigens and antigens associated with the erythroid or myeloid lineage (3,16–20), in addition to the light scattering classification.

Bone marrow samples in the B category are characterized by a uniform cell population, whereas leukemias in the C category are heterogeneous with cells differentiating into separate lineages as indicated by light scattering. This is in fact discernible in leukemias of FAB subgroups M2, M4, and M5. FAB M2 is characterized by the partial maturation of the leukemic cell population along the myeloid differentiation pathway. By light scattering categorization, the majority of these bone marrow aspirates fall into the heterogeneous groups C1 and C2. Six of 14 samples of the FAB subgroup M5 were also grouped in categories C1 and C2, whereas the majority exhibited a uniform light scattering profile with the lowest granularity in the B category (B1). Leukemic samples classified as M4 by light microscopy and cytochemistry distribute equally over the B and C subcategories. Based on light scattering, two unique subgroups were identified (B2, C3) in which the majority of the cells had large orthogonal light scattering indicative of higher granularity and thus relative mature differentiation features. The ANLL FAB M3 (promyelocytic) clustered in subgroup B3 in which only one cell cluster was predominant with large forward light scattering, whereas subgroup C3 only included M4 leukemias and both eosinophilic leukemias (M4eo).

An advantage of the automated analysis of 10 000 or more cells is the additional identification of minor subgroups with abnormal light scattering features which were found in 60% of the leukemias. Whether the cells in these abnormal light scattering locations are part of the leukemic cell clone can only be answered by their isolation and subsequent analysis using additional markers of clonality such as aberrant antigen expression, genetic abnormalities, or isoenzyme pattern.

FAB classification is not prone to error. In a recent study by the Children’s Cancer Study Group of 486 newly diagnosed myeloid leukemias, the concurrence of different cytologists averaged only 73% (21). The light scattering profiles showed a clear relationship with the FAB subgroups providing ‘objective’ criteria for morphological leukemia classification by which the discordance between different cytologists can be decreased and the less experienced hematologists supported. The categorization of acute leukemias by light scattering offers the possibility for a combined evaluation of antigenic profile, i.e. immunophenotype and cell morphology. The leukemic cell population can be identified and the expression of cell surface antigens within this specific cell population can be assessed. The combination of various parameters simultaneously measured on the leukemic cells will help in the assessment of ‘true’ aberrant antigen expression, subclassification of acute leukemias, and the identification of heterogeneity within each leukemia.

REFERENCES
