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FLOW CYTOMETRIC ANALYSIS AS A QUICK AND EFFICIENT METHOD FOR MORPHOLOGICAL CHANGES DETERMINATION IN HEMATOLOGICAL DISEASES. RBCs OF GAUCHER DISEASE AS A MODEL

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ABSTRACT. Flow cytometry is a general method for the rapid individual analysis of a large numbers of cells using light-scattering, fluorescence and absorbance measurements. The forward-scattered light provides informations about the size of the cells and can be detected without further manipulation. The sideways-scattered light is affected by several parameters, including granularity, cell size and cell morphology. Gaucher disease (GD) is a sphingolipidosis caused by a deficiency of the enzyme glucocerebrosidase and enhanced erythrophagocytosis is one feature of the disease indicating abnormal macrophage-RBC interactions. The aim of this study was to compare flow cytometric analysis with the morphology obtained by scanning electron microscopy analysis (SEM) of RBCs sampled from seven untreated type 1 GD patients and two of the same patients who then underwent nine months of enzyme replacement therapy (ERT). The results provide that flow cytometric analysis can be a quick and efficient method in haematological diseases diagnosis and monitoring.

Keywords: flow cytometry, scanning electron microscopy, erythrocytes, Gaucher disease, morphological changes, medical monitoring

INTRODUCTION

In flow cytometry, single cells or particles pass through a laser beam in a directed fluid stream. The interaction of the cells with the laser beam – their absorption, scattering, and/or fluorescence – can be monitored for each individual cell. The forward-scattered light provides information on the size of the cells and can be detected without further manipulation. The sideways-scattered light is affected by several parameters, including granularity, cell size and cell morphology. These data can be correlated with different cell characteristics and cell components, and thus, distributed data about a cell population can be obtained easily.

Flow cytometry was first used in medical sciences such as oncology (e.g., for diagnosis of cancer, chromosomal defect diagnosis) and hematology and subsequently in biology, pharmacology, toxicology, bacteriology, virology, environmental sciences, and bioprocess monitoring. Medical and clinical applications of flow cytometry still account for the vast majority of publications on this technique (Métézeau et al., 1988, Ronot et al., 2006).

Gaucher disease (GD) is a sphingolipidosis due to glucocerebrosidase deficiency. The accumulation of glucosylceramide, unambiguously identified in 1953 by paper chromatography (Montreuil et al., 1953) in macrophages leads to the formation of Gaucher cells, the hallmark of the drsease. Anemia is a frequent feature of the condition and abnormal enhanced phagocytosis of red blood cells (RBCs) has been recurrently reported (Sharpe et al., 2009, Bitton et al., 2004, Mignot et al., 2006).

Based on their properties we hypothesized that the light scattering measurement, forward-scatter and side-scatter (FSC and SSC) can provide easier information on cell morphology and morphological changes that may occur in some pathology. To investigate this, we compared flow cytometric analysis with scanning electron microscopy (SEM) to study the morphology of RBCs sampled from seven untreated type 1 GD patients and from two of the same patients who then underwent nine months of ERT.

MATERIALS AND METHODS

Patients' characteristics

Patients G1 to G7 were diagnosed with type 1 GD by means of clinical findings, low glucocerebrosidase activity and mutations in the GBA gene. All were male and aged 2.5 to 16 years at the time of the study. Six of

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them had a splenomegaly and thrombocytopenia without neurological, skeletal or pulmonary signs of the disease. Anemia was present in two patients. Gaucher disease was diagnosed in patient G5 after a second episode of osteonecrosis. Clinical examination of this patient revealed no splenomegaly and blood cell count disclosed normal platelet and hemoglobin levels. All patients were naïve to ERT at the time of study. Their main characteristics are reported in Table 1. Patients G6 and G7 are siblings and their RBCs were studied before and after nine months of treatment with ERT (Cerezyme 60u/kg/2weeks).

Biological materials

Blood samples were collected from patients and healthy donors into heparinized tubes. Cells were sedimented by centrifugation, 350g at 4°C for 5 min. After the removal of plasma, platelets and leukocytes by pipetting, RBCs were washed three times in Dulbecco's phosphate buffered saline solution (PBS; pH 7.4; 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na2HPO4, and 1.5 mM KH2PO4).

Flow cytometric analyses

Flow cytometric analyses were performed on a FACScalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) using the CellQuest software for acquisition and analysis. The light-scatter channels were set on linear gains and a minimum of 10,000 cells being analyzed.

Scanning electron microscopy (SEM) analysis

Erythrocytes were fixed for 4 h with a 1.25% glutaraldehyde solution in 0.1 M sodium cacodylate buffer pH 7.2 and post-fixed for 4 h in 1% osmium tetraoxide in the same buffer. The suspensions were then filtered onto 0.2 µ Anodisc filters and dehydrated in an ethanol series. After drying with carbon dioxide by the critical point method and sputter-coating with gold, samples were examined on a 35 CF JEOL SEM.

RESULTS

Morphological changes of Gaucher RBCs

Flow cytometric analyses. As shown in figures 1 and 3, flow cytometric analyses revealed significant morphological changes of Gaucher RBCs in all untreated patients (Fig. 1). Values for the geometric mean XGeoMean, proportional to cell diameter, varied widely among patients from 157 (patient G2) to 354 (patient G6a) when compared to that for normal RBCs, i.e. 313 ± 28 (Fig. 3).

In the same way, values for YGeoMean proportional to internal granularity, varied from 213 (patient G2, Fig. 1) to 301 (patient G4, Fig. 1) when compared to that for normal RBCs, i.e. 298± 25 (Fig. 3). Also see that YGeoMea values are consistent with biochemically determined levels of Hb (Table 1) and that flow cytometric analysis can replace it.

These morphological changes were no longer observed in patients G6 and G7 after a nine month-long treatment with ERT, as shown in Figure 1 and 3 (panels G6b and G7b).

Table 1

Hemoglobin level of Gaucher patients

Patients	G1	G2	G3	G4	G5	G6a	G6b	G7a	G7b
Age (y)	12.5	4.8	2.5	14.5	16	9.3	10.5	5.3	6.5
Hb (g/dl)	11.3	6.9	10.8	13.5	13.9	9.2	11.3	11.8	12.3

Scanning electron microscopy analyses

SEM disclosed numerous cell aggregates (not shown) and a variety of dysmorphic RBCs in amongst discocyte forms in all patients (Fig. 2) that were not found in control samples, hence confirming the data obtained with flow cytometric analyses. Many of the observed cells displayed the morphological characteristics of echinocytes, schistocytes, ovalocytes and dacryocytes (teardrop cells), and a few more had very strange shapes including some apparent three-sided cells resembling knizocytes. Dacryoytes were not found in patients G4 and G5, however. This tendency toward poikilocytosis was no longer observed nine months after the start of ERT in patients G6 and G7.

DISCUSSIONS

In flow cytometry, the laser beam is split to give both exact cell size determination via transmission measurement and velocity determination. When it impacts a cell, the excitation light is scattered in both forward and sideways directions. The forward-scattered light provides information on the size of the cells and can be detected without further manipulation. The sideways-scattered light is affected by several parameters, including granularity, cell size and cell morphology.

Focusing on the RBC side of the crosstalk, we investigated Gaucher RBCs by means of flow cytometric analyses and SEM.

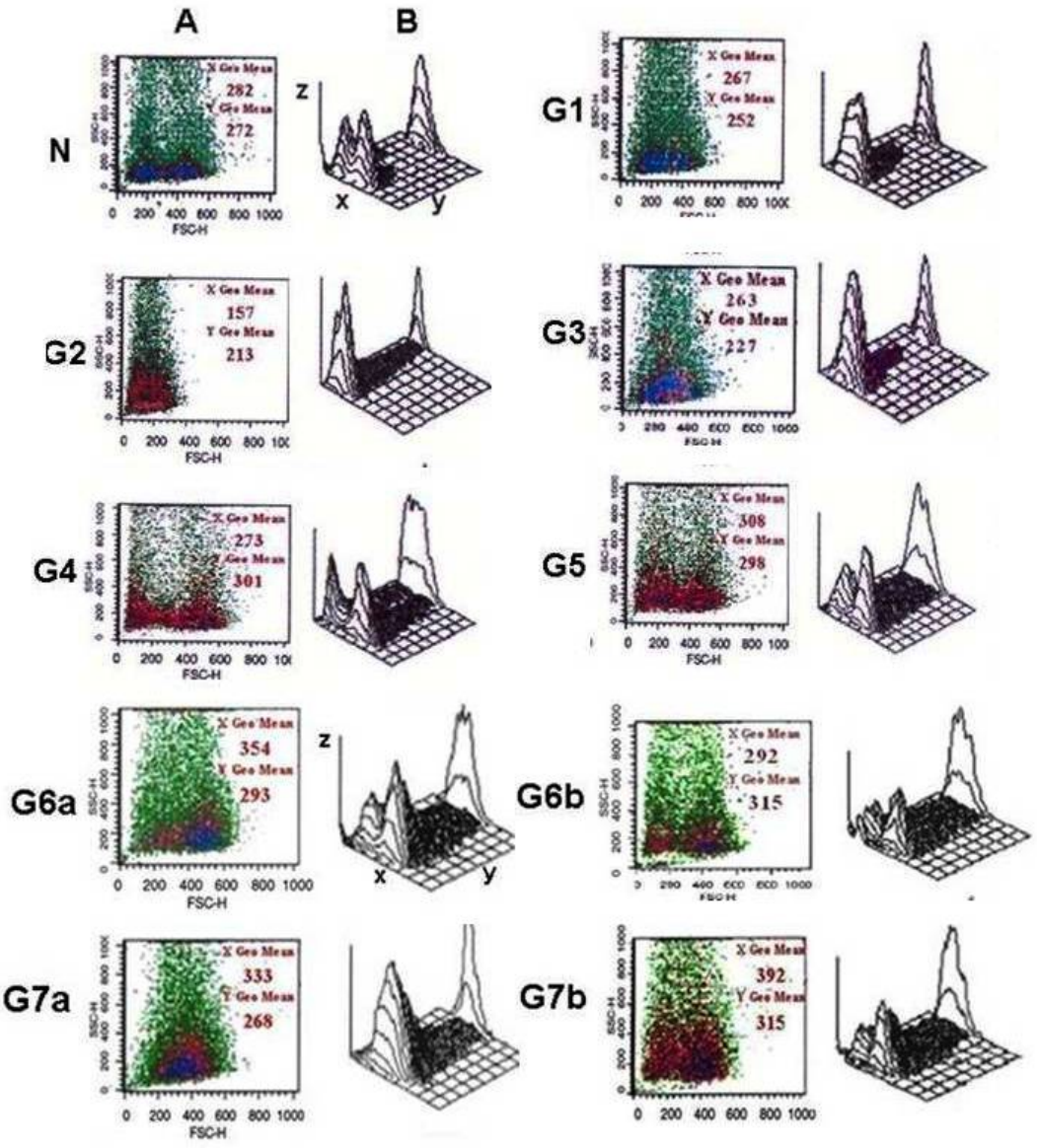

Fig. 1 Bidimensional (A) and tridimensional (B) representation of dot-plot analyses in the mode FSC/SSC, of normal (N) and untreated Gaucher erythrocytes (G1 to G5) and from patients G6 and G7 before (g6a-g7a) and after (g6b-g7b) nine months of ERT. Abcissae and x: forward scatter (cell size); ordinates and y: side scatter (cell density); z: cell number. Data are representative of three analyses giving similar results.

Surprisingly we observed discocytes mixed with variable amounts of morphologically abnormal RBCs. This dramatic change in the morphology of Gaucher RBCs was first suggested by flow-cytometric analyses revealing important variations in the size/density distribution of RBC populations in 4/7 untreated GD patients compared with RBCs from control or treated patients. This was confirmed by SEM in all patients with an abnormal dot-plot. In untreated patients with a normal dot-plot some abnormal RBCs were observed, though discocytes largely predominated. Dot-plot anomalies were therefore not due to artifactual preparation-related changes and the flow-cytometric analyses performed in this study were sensitive enough to predict the RBCs mean morphology. Likewise, the normalization of the dot-plot profile of the two patients subsequently treated with ERT was associated with a normalization of RBC shape observed by SEM. The analysis of RBCs from another Gaucher patient who had received treatment with ERT for 11.5 years also gave a normal dot-plot (not shown). It is reasonable therefore to conclude that ERT is the likely cause of recovery of RBC morphology. Many abnormally shaped RBCs observed in our study resembled schistocytes, equinocytes and teardrop erythrocytes. The morphological changes may lead to be the sequestration of RBCs in spleen capillaries and their subsequent remoral from the circulation.

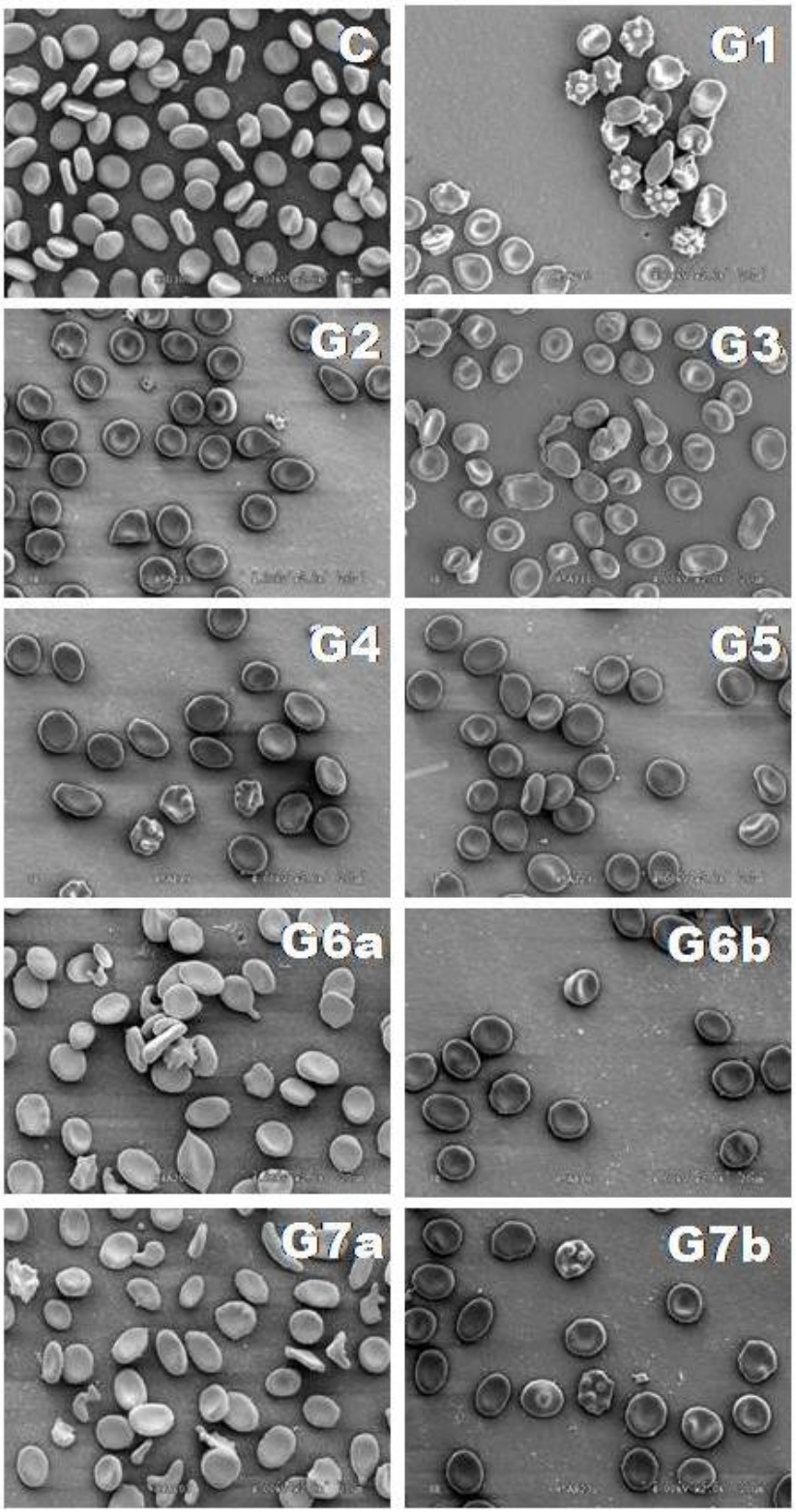

Fig. 2 SEM analyses of normal (N) and untreated Gaucher erythrocytes (G1 to G5) and from patients G6 and G7 before (g6a-g7a) and after (g6b-g7b) nine months of ERT

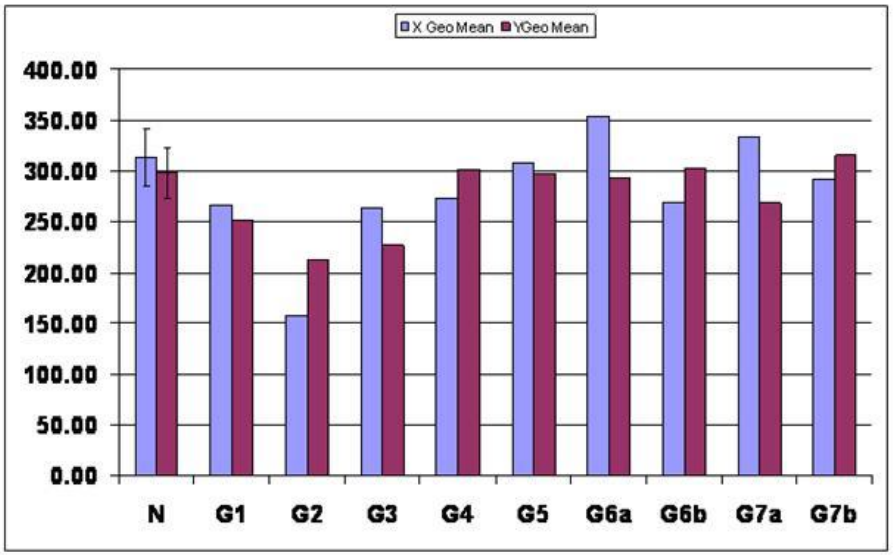

Fig. 3 Histogram of X Geo Mean and Y Geo Mean values of normal (N) and Gaucher (G) RBCs G1 to G7 refer to dot-plot analyses of Figures 1

CONCLUSIONS

Our present study provides that flow cytometric analysis using light-scattering measurements can be a quick and efficient method for morphological change determination in hematological diseases diagnosis and monitoring after treatment. Through the use of light-scattering, fluorescence, and absorbance measurements on cells, a very wide range of cellular parameters can be measured with flow cytometry. Although many of the same measurements can be made without the use of this equipment, flow cytometry remains the only way to obtain information on how the parameter is distributed in the population.

In conclusion, this promising method seems to be a very convenient method in the diagnostic work-up to obtain a sensitive detection of morphological change and for further improvement in diagnostic power.

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