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Original paper

Cytometric analysis of erythrocytes in different types of anemia – a tool for clinic and medical biotechnology industry

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Abstract

Anemia is one of the most common disorders in the world generated by multiple causes. Despite a good understanding of the causes of anemia, there is still uncertainty about how best this should be investigated, prevented and managed. This reflects the limitations of laboratory tests, as well as the poor understanding of the complex physiological mechanisms. A better understanding of the nature of anemia and the changes in erythrocytes may provide new strategies to benefits of patients.

In this study, it was identified by flow cytometric and SEM analysis, the morphological changes of forms that deviate from the classical discoid shape, which had an increase in the mean fluorescence intensity of calcein (erythrocyte viability measured by the level of intracellular esterase activity with the Calcein-AM method). The results obtained for RBCs from the patients with different types of anemia did not demonstrate a massive phosphatidylserine externalization measurable with Annexin-V-FITC compared to the values for normal erythrocytes. The present study provides that flow cytometric analysis can be a quick and efficient method for diagnosing in haematological diseases and monitoring and in the medical biotechnology industry in order to develop drugs to treat anemia.

Keywords Anemia, erythrocytes, eryptosis, flow cytometry, scanning electronic microscopy, biotechnology.

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Introduction

Anemia is a worldwide problem (24.8% of the world population) that affects patients of all ages and it is the most common pathology of the blood, being produced by different underlying causes (WORLD HEALTH ORGANISATION [1], TURNER & BHIMJI [2]). The World Health Organization (WHO) defines anemia as a haemoglobin concentration of less than 12 g/dl in females and less than 13 g/dl in males (WHO [3]). According to these criteria, 10 to 20 percent of women and 6 to 30 percent of men above the age of 65 years are anemic. According to the National Institutes of Health's National Heart, Lung, and Blood Institute, there are over 400 types of anemia (NATIONAL HEART LUNG AND BLOOD INSTITUTE [4]). In general, anemia can be classified in many ways, considering the morphology of red blood cells (RBCs), discernible clinical spectra and underlying etiologic mechanisms. Anemia is a condition of low haemoglobin (Hb) and it can be subdivided several different ways: symptomatic versus non-symptomatic, or more frequently, by laboratory findings such as macrocytic versus microcytic or normochromic versus hypochromic (TURNER & BHIMJI [2]).

The three main classes include high amount of blood loss (acutely – such as a haemorrhage or chronically – through low-volume loss), excessive destruction of red blood cells (haemolysis) or deficient erythrocytes production by ineffective haematopoiesis (UTHMAN [5], WICK et al [6], BRIDGES & PEARSON [7]).

Anemia is a frequent feature of certain conditions and there has been reported abnormal phagocytosis of red blood cells (RBCs) (BITTON et al [8], MIGNOT et al [9], BRATOSIN et al [10]). The removal of senescent or abnormal RBCs, now regarded as a particular apoptotic phenomenon (programmed cell death – PCD), demonstrated for the first time by Bratosin and collaborators (BRATOSIN et al [11], BRATOSIN et al [12], BERG et al [13], BRATOSIN et al [14]) and called *erythroptosis* (DAUGAS et al [15]), or more recently *eryptosis* (LANG et al [16]), involves macrophagic phagocytosis–triggering signals (like the desialylation of glycoproteins, a signal for the capture of RBCs and the externalization of phosphatydylserine, a signal for their phagocytosis) that are exposed (BRATOSIN et al [11]).

Many changes of the properties of erythrocytes, including morphological changes, facilitate their sequestration inside the blood vessels of the spleen and their phagocytosis by splenic macrophages (MOHANDAS & GALLAGHER [17], BRATOSIN et al [18]).

Flow cytometry supplies a technology both to evaluate the morphology of cells and to quantify the labelling intensity and signal distribution of many fluorescent markers on a single cell basis with high yield in a statistically robust way, and thus is ideally suitable for the study of cell biology. Used first in oncology and haematology, flow cytometry is today used in all biological sciences (COMBRIER et al [19]). Medical and clinical applications of flow cytometry still account for the vast majority of publications on this technique (MÉTÉZEAU et al [20], RONOT et al [21]).

In the literature it was discussed that based on the longevity of erythrocytes, the measurement of individual red cells, rather than determining mean red cell indices of the whole erythrocyte population, should allow a rapid identification of circulating sub-populations (BRATOSIN et al [22], BRATOSIN et al [23]) or, for example, hypochromic and/or microcytic cells, as they are produced by an iron-deficient erythron (SCHAEFER & SCHAEFER [24]).

More recently, flow cytometric methods have been developed to determine red cell volume either by measuring changes in electrical resistance (KUBITSCHEK [25]) or by measuring the amount of light scattered by individual red cells (BRATOSIN et al [22], BRATOSIN et al [23]). Modern haematology analyzers are capable of simultaneously and independently measuring both volume and hemoglobin concentration in individual sphered red blood cells using monochromatic light at two different angular intervals (MOHANDAS et al [26], TYCKO et al [27]). King et al, 2000, used the flow cytometric test to measure the fluorescence intensity of intact red cells labelled with the dye eosin-5-maleimide, in patients with haemolytic anemia (KING et al [28]).

Based on all those mentioned, 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 of different types of anemia. We also investigated the degree of phosphatidylserine externalization and erythrocyte viability according to our original method devised with of Calcein-AM.

Materials and Methods

1. Patients' characteristics

22 patients hospitalized at Arad Clinical Emergency County Hospital were divided into 3 groups depending on the underlying disease, such as: 1) patients with iron deficiency anemia (9 women with a mean age of 59.25 years and 3 men with a mean age of 66.00 years), 2) patients with autoimmune haemolytic anemia (2 patients, both women with a mean age of 75.00 years) and group with plurifactorial anemia (8 patients, all female, with a mean age of 67.75 years.). The control group was composed of 8 subjects, healthy individuals, four females and four male with a mean age of 47.00 years. The diagnosis of anemia was based on WHO standards, 2001 (WORLD HEALTH ORGANIZATION [29]). The diagnosis and treatment protocol followed was the one developed within the Hematology Department in Arad County Emergency Hospital. In this study, patients with blood transfusions were not evaluated and blood samples were collected at the onset of hospitalization.

2. Biological materials

Blood samples were collected from patients and healthy donors into heparinized tubes and tested immediately after sampling. Cells were sedimented by centrifugation, 350g at 4°C for 5 min. for the removal of plasma, platelets and leukocytes by pipetting, RBCs were washed three times in Dulbecco's phosphate buffered saline solution, pH 7.4 and analyzed by flow cytometry (MITROFAN-OPREA et al [30], BRATOSIN et al [14]) and Scanning Electron Microscopy.

3. Chemicals

Fluorescein conjugated annexin-V (Annexin-V-FITC), HEPES binding buffer (HEPES buffer pH 7.4 containing 2.5mM calcium chloride), were obtained from Pharmingen (San Diego, CA, USA and Calcein-AM from Sigma Aldrich (St. Louis, M0, USA).

4. Flow cytometric analyses

Flow cytometric analyses were performed on a Cytomics FC 500 Beckman Coulter equipped with an argon-ion excitation laser, 488 nm, using the CXP software for acquisition and analysis. Cells were gated for the light scatter channels on linear gains, and the fluorescence channels were set on a logarithmic scale.

Morphological changes assessment of red blood cells by light scattered measurements

Analysis of the scattered light by flow cytometry in the mode FSC/SSC provides information about the cell size and structure. The intensity of light scattered in a forward direction (FSC) correlates with cell size and the intensity of scattered light measured at a right angle from the laser beam (SSC) correlates with granularity, refractiveness and presence of intracellular structures that can reflect the light, being associated with cell shrinkage. RBCs suspension in isotonic PBS buffer, pH 7.4, was gated under forward and side scatter parameters (FSC versus SSC).

Flow cytometric analysis of phosphatidylserine exposure

Phosphatidylserine exposure on RBCs was assessed using Annexin-V-FITC. Erythrocytes were suspended (10^6 cells) in HEPES buffer pH 7.4 containing 2.5 mM calcium chloride with 10 µl (0.1 µg) of Annexin-V-FITC solution and incubated for 15 min at room temperature in the dark. The cells were gated for biparametric histograms FL1 (FITC fluorescence) versus FL2 (RBCs autofluorescence).

Flow cytometric measurement of cell viability using Calcein-AM

Cell viability assessment was studied according to the procedure of Bratosin et al, 2005, which is based on the use of acetoxymethyl ester calcein (Calcein-AM) (BRATOSIN et al [31]). RBCs (4 x10^5) in PBS buffer, pH 7.4 were incubated with Calcein-AM at 5 µM final concentration in Calcein-AM, for 45 min at 37°C in the dark and the samples were immediately analysed for calcein fluorescence retention in cells.

5. Microscopic analysis

Scanning Electron Microscopy (SEM) analysis.

Fresh erythrocytes were fixed for 4 hours in a 1.25% glutaraldehyde in cacodilate buffer (0.1 M, pH of 7.2), washed 3 times with distilled water, filtered through onto 0.2µm Anodisc filters and analyzed at a Hitachi SU1510.

Results and Discussions

1. Morphological changes analyses of RBCs anemia by scanning electron microscopy (SEM) and flow cytometry

Red blood cells from different types of anemia RBCs are generally characterized by their abnormal rheological properties. Focusing on the RBCs morphology, we investigated RBCs by means of flow cytometric analyses and SEM.

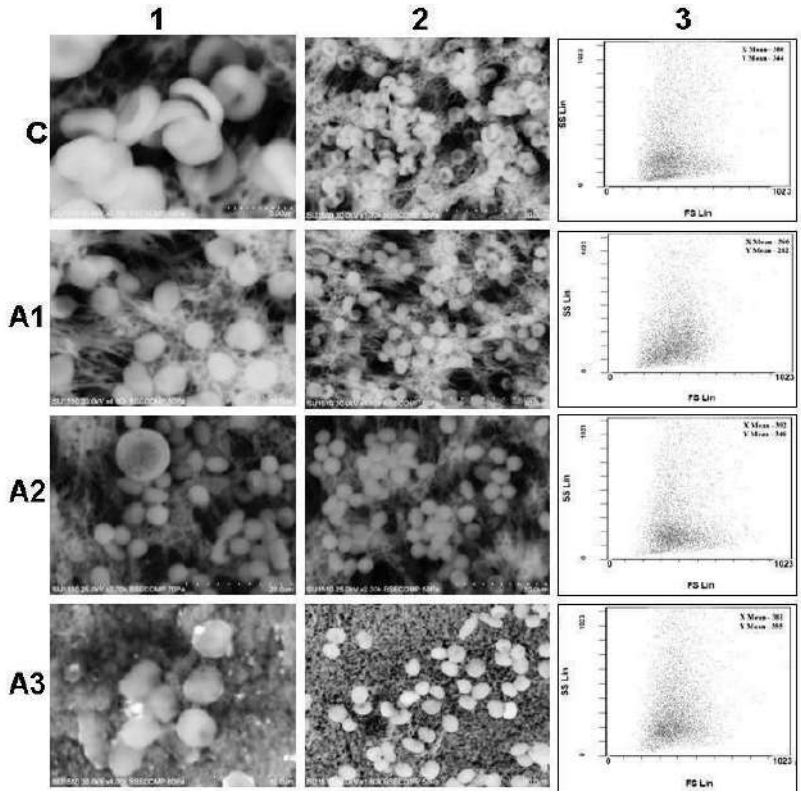

Figure 1. Electronic Scanning Microscopy (SEM) analysis (1, 2) and dot-plot analysis (3) of erythrocytes from different types of anemia compared with normal RBCs **(C); A1.** patient male, 65 years diagnosed with severe iron deficiency anemia; **A2.** female patient, 71 years diagnosed with autoimmune hemolytic anemia; **A3.** female patient, 60 years with severe anemic syndrome of unspecified etiology. Data shown are representative for similar results.

As shown in Figure 1, a first analysis by scanning microscopy of the anemia RBCs compared with flow cytometric analyses revealed significant morphological changes in all 22 patients with respect to the control cells. Normal human erythrocytes have a biconcave discoid shape, whereas erythrocytes from different types of anemia are much smaller, mostly with a spherical morphology. These morphological changes have led to the dot-plot different from that obtained for the control erythrocytes.

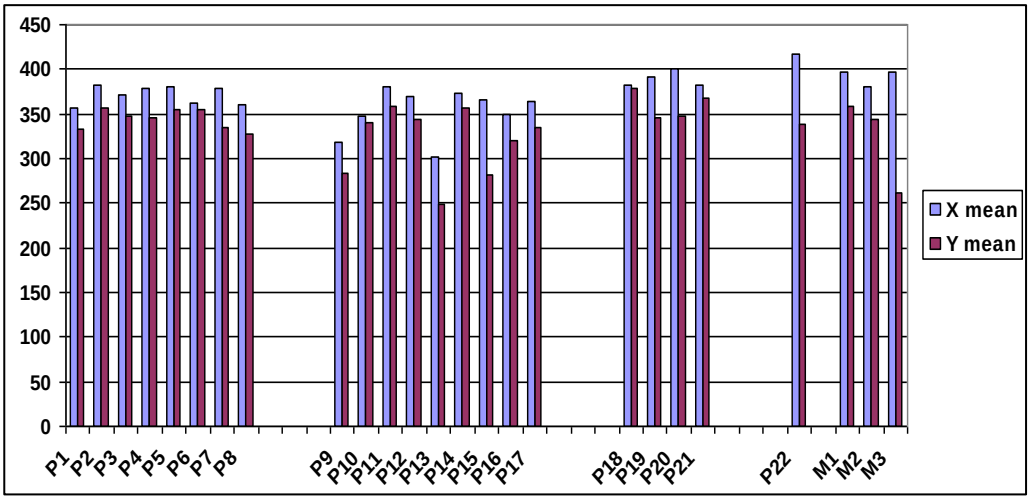

Figure 2. Histogram of X mean and Y mean values of normal (M1-M3) and Multi-factorial anemia, anemic or non-diagnosed syndrome (P1-P8), Severe iron deficiency anemia (P9-P17), Hemolytic anemia (P18-P21) and Macrocytic anemia (P22) RBCs.

Values for the Xmean, proportional to cell diameter, varied widely among patients (Figure 2) depending on the type of anemia, from 301 (patient P13) to 417 (patient P22) when compared to that for normal RBCs (an average of 388± 8). In the same way, values for Ymean proportional to internal granularity, varied from 249 (patient P13) to 368 (patient P21) when compared to that for normal RBCs, i.e. 321±43 (Figure 2). Also see that Ymean values are consistent with biochemically determined levels of Hb (data not shown) and that flow cytometric analysis can replace it.

2. Flow cytometric analysis of phosphatidylserine exposure

Phosphatidylserine (PS) exposure on the outer leaflet of plasma membrane is regarded as one of the signals allowing macrophages to ingest erythrocytes (BRATOSIN et al [32]). Consequently, was evaluated the phosphatidilserine exposure by flow cytometric analyses usig annexin-V-FITC labelling on red blood cells from different types of anemia compared with normal RBCs (Figure 3).

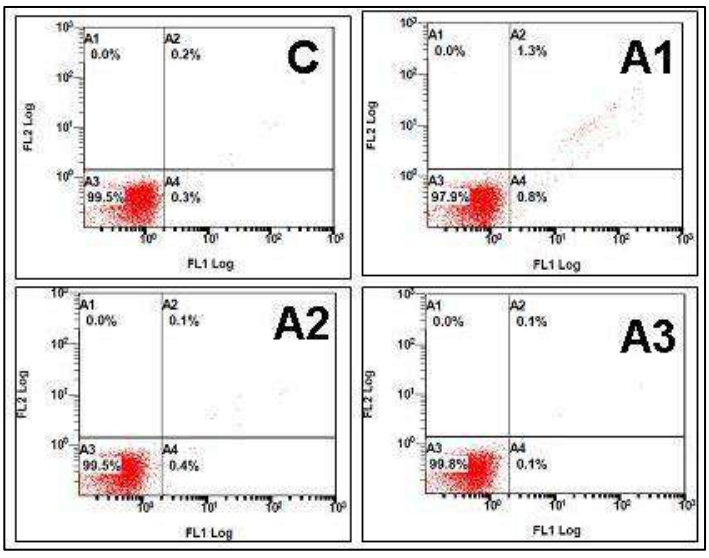

Figure 3. Flow cytometric quadrant analysis of phosphatidylserine exposure (Annexin-V test) of erythrocytes from different types of anemia compared with normal RBCs **(C)**; **A1.** patient male, 65 years diagnosed with severe iron deficiency anemia; **A2.** female patient, 71 years diagnosed with autoimmune hemolytic anemia; **A3.** female patient, 60 years with severe anemic syndrome of unspecified etiology. FL1: annexin-V fluorescence. FL2: red autofluorescence. Lower left quadrant: viable annexin-V negative cells; lower right quadrant: annexin-V positive cells. %: percentages of different regions of varying fluorescence intensity. Number of counted cells: 10,000. Data shown are representative for similar results.

The results obtained for erythrocytes from the patients with different types of anemias did not demonstrate a massive PS externalization, measurable with Annexin FITC, compared to the values obtained for normal erythrocytes. If for normal erythrocytes, the % of erythrocytes Annexin positives varied between 0.5% ± 0.2%, in the case of anemia, this percentage was similar or at most 2.1% for the RBCs from a severe iron deficiency anemia. The percentage of low positive annexin erythrocytes is easily explained by the fact that once outsized phosphatidylserine residues to the cell surface, RBCs are immediately captured and phagocyte macrophages, which make the % of annexin positive cells to be approximately small, but the removal rate of the circulation may be very high.

3. Flow cytometric measurement of cell viability using Calcein-AM

Measurement of erythrocytes viability is very difficult, being free of nucleus and organelles. In 2005, the research group of Bratosin have developed a new flow cytometric assay for the measurement of cells viability using Calcein-AM (BRATOSIN et al [31]).

The assay is based on the use of acetoxymethyl ester of calcein (calcein-AM), a nonfluorescent fluorescein derivate that is converted by cytosolic esterases into green fluorescent calcein which is retained by cells with intact membranes.

In this regard, it is important to mention that it was previously demonstrated that in old RBCs calcein fluorescence was lower than that in young RBCs due to decreased esterase activity. Similarly, it was observed a good correlation with the ATP content, which was lower in old RBCs than in young ones, and also, the decreased calcein fluorescence intensity of old RBCs was not associated with annexin-V staining, suggesting that decreased esterase activity precedes phosphatidylserine exposure (BRATOSIN et al [31]).

In this context, application of this assay, as shown in Figure 4, surprisingly we observed that all patients showed an increase in the mean fluorescence intensity (MFI) of calcein varying from MFI-227 to MFI-839.

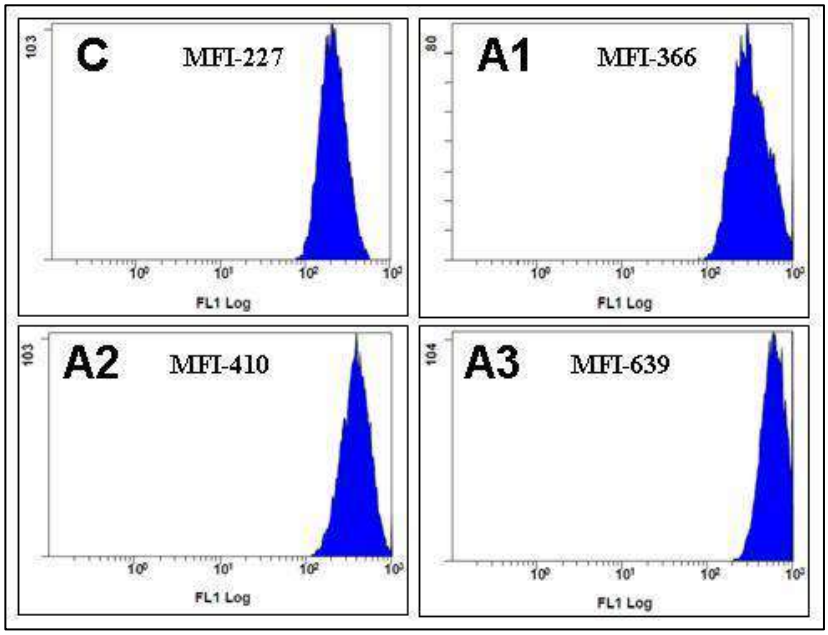

Figure 4. Flow cytometric analyses of cell esterase activity (calcein-AM viability test) of normal RBCs **(C)** and of erythrocytes from different types of anemia; **A1.** patient male, 65 years diagnosed with severe iron deficiency anemia; **A2.** female patient, 71 years diagnosed with autoimmune hemolytic anemia; **A3.** female patient, 60 years with severe anemic syndrome of unspecified etiology. Numbers represent fluorescence mean values (MFI). Number of counted cells: 10,000. Data shown are representative for similar results.

For patients with iron deficiency anemia, MFI ranged between MFI-277 for a slight deficiency anemia to MFI-839 for a severe iron deficiency anemia. Average value for 9 patients diagnosed with iron deficiency anemia it was MFI-582. In the case of autoimmune hemolytic anemia, calceine fluorescence of erythrocytes ranged between MFI-345 to MFI-873 for a severe autoimmune hemolytic anemia. Average value for 4 patients diagnosed with autoimmune hemolytic anemia it was MFI-548. Also, in the case of patients with plurifactorial anemia, MFI was higher than MFI for normal erythrocytes, varying between MFI-253 and MFI-454. The viability of erythrocytes from all 7 patients with anemia of unspecified origin (unknown origin), MFI was also bigger than MFI for control erythrocytes, varying between MFI- 502 and MFI-697.

As it was already mentioned, Calcein-AM generally has a higher fluorescence in younger cells than in the elderly cells. In the case of anemias, increased esterase activity can be explained by an increased erythropoiesis, known for the compensation of the loss in the blood of

red blood cells. Taking into account that until now is not elucidated the role of intracellular erythrocyte esterases, the present results cannot be fully interpreted, and further investigations should be performed.

Conclusion

This study is based on previously overlooked morphological changes in RBCs from patients with different types of anemias, and it was found that these morphological changes, possibly together with a high percentage of erythrocytes that externalize phosphatidyl-serine (difficult to quantify ex vivo) may collectively favour erythrophagocytosis and hence play a causal role in the enhanced splenic clearance of RBCs (BRATOSIN et al [18]), finally generating anemia.

The unexpected results on increased esterase activity in all patients with anemia are very interesting and must be further investigated to elucidate this enigma and the role of intracellular esterases in the life of erythrocytes.

Moreover, the present study provides that flow cytometric analysis using light-scattering measurements and fluorescence can be a quick and efficient analytical technique in the diagnostic work-up to obtain a sensitive detection of the morphological changes and for further improvement in diagnostic power.

In conclusion, flow cytometry seems to be a very convenient and promising method in the clinic and medical biotechnology industry to develop drugs for anemia treatment.

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