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FLOW CYTROMETRIC ANALYSIS OF RED BLOOD CELLS IN POLYCYTHEMIA VERA

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ABSTRACT. Polycythemia vera (PV) is characterized by an absolute increase in the red blood cell mass, but the mechanisms are not completely understood. In this study, we identified by flow cytometric analysis morphological forms that deviate from the classical discoid shape, who had a more viability determined by Calcein-AM method and a normal phosphatidylserine exposure level. Measurement of glycoconjugate sialylation using lectines demonstrates a low degree of sialilation of membrane glycoconjugates of Polycythemia vera RBCs but which is close to normal after treatment and an increased percentage of cells with active caspase-8 and -3 compared to normal RBCs, showing that the organism tries to restore the apoptotic mechanism for maintaining the normal hematocrit. Our observations may contribute for understanding the survival of RBCs in Polycythemia vera and may also participate in elucidation of the mechanism in pathogenesis of this disease.

Keywords: polycythemia vera; PV; rbcs, red blood cells; erythrocyte viability; phosphatidylserine exposure; caspase-3; caspase-8; Annexin-V; Calcein-AM; flow cytometry

INTRODUCTION

Polycythemia is literally translated as "many cells in the blood". Only erythrocytosis (an alternative term for these disorders) produces polycythemia since leukocytes and platelets are present in blood in far smaller proportions. This disease may be due to increased proliferation or decreased apoptosis of erythroid progenitors or to delayed erythroid differentiation with an increased number of progenitor cell divisions (Prchal J.T., 2001). Polycythemia vera (PV) is a malignant disorder of hematopoietic stem cells which is characterized by clonal myeloproliferation with increased production of morphologically normal mature red blood cells, white cells and platelets (Berlin, N.I., 1975; Adamson *et al.*,1976; Spivak, J.L. 2002; Bai *et al.*,2004). First described in 1892 by Vaquez (Vaquez H.,1892) Polycythemia vera is a disease with an incidence of at least 2 per 100 000 and is a trilineage hematopoietic cell hyperplasia (Berglund, S. & Zetterval O.,1992; Mcnally, *et al.*,1997). Prolonged red cell survival, another theoretical cause of polycythemia, has not yet been demonstrated (Berlin *et al.*, 1951; Fernandez-Luna *et al.*, 1998). Recent investigations have focused on a number of molecules involved in signal transduction pathways mediated by erythropoietin (Epo) and other growth factors, but human erythroid malignancies (PV and erythroleukemia) are associated with erythropoietin-independent growth and differentiation (Carneskog *et al.*,1998; Ugo *et al.*,2004). Despite recent advances in the characterization of the malignant PV clone, the molecular mechanism and the abnormalities associated with the development of this disorder remain unknown (Spivak, J.L., 2002). Regarding these controversed results, we considered of interest to analyse and characterise red blood cells in Polycythaemia vera regarded as cancerous cells by applying methods for characterisation of apoptotic cells. Cell viability and death were analyzed by flow cytometry, a method ideally adapted for the study of cell death and for rapid and individual analysis of a large number of cells.

MATERIALS AND METHODS

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), Calcein-AM from Sigma Aldrich (St. Louis, M0, USA) and CaspGLOW^{TM} Fluorescein active caspase-8 and caspase-3 staining kits from BioVision Research Products (Mountain View, CA, USA). Fluoresceinylisothiocyanate lectins (FITC-lectins): MAA (*Maackia amurensis* agglutinin), SNA (*Sambucus nigra* agglutinin) and RCA_{120} (*Ricinus communis* agglutinin) were from EY Laboratories (San Mateo, CA, USA).

The flow cytometer was a Becton-Dickinson FACScan apparatus (San Jose, CA, USA) with CellQuestPro software for acquisition and analysis.

Isolation of erythrocytes

Human blood was taken up on heparin from the patients with PV before and after treatment. The diagnosis of the disease was established according to commonly accepted clinical and laboratory criteria (Berk *et al.*, 1986). The blood samples were washed

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thrice by centrifugation (5 min, 1000 x g at 4°C) with phosphate buffered saline (PBS):Na_2HPO_4 1.8 mM, KH_2PO_4 140 mM, NaCl 2.7 mM, KCl, pH 7.4. After centrifugation, plasma, platelets and leukocytes were removed by aspiration and the red blood cells were resuspended (10^7 cells per ml) for further experiments in isotonic phosphate-buffered saline (PBS) solution pH 7.4.

Flow cytometric analysis

Flow cytometric analyses were performed on a FACScan cytometer using CellQuestPro software for acquisition and analysis. Cells in suspension in isotonic PBS buffer pH 7.4 were gated for the light scatter channels on linear gains, and the fluorescence channels were set on a logarithmic scale with a minimum of 10,000 cells analyzed in each condition.

Morphological changes assessment of red blood cells in Polycythemia vera by light scattered measurements

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

Flow cytometric measurement of the binding of fluorescein isothiocyanate (FITC)-labeled lectins to RBCs

According to the protocol described by Bratosin (Bratosin *et al.*, 1995), a solution (50µl) of FITC-labeled lectins in phosphate buffered saline (PBS)-phenylmethysulfonyl fluoride (PMSF) buffer, pH 7.4 (10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 140 mM NaCl, 2.7 mM KCl, 0.2 mM PMSF), was added to 50 µl of a red blood cell suspension in the same buffer (corresponding to $2x10^6$ red cells). After 1h incubation at 4°C in the dark, 10,000 cells were analyzed directly. The binding for each lectin was first studied at concentrations ranging from 0 to 50 mM in order to determine the optimal lectin concentration. This experimental protocol was applied to the following FITC-labeled lectins: *Sambucus nigra* agglutinin (SNA) and *Maackia amurensis* agglutinin (MAA), specific for sialic acids and *Ricinus communis* agglutinin (RCA_{120}) specific for β-galactosyl terminal residues. Experiments were performed at least three times with three replicates each time.

Flow cytometric measurement of cell viability using Calcein-AM

Cell viability assessment was studied according to the procedure of Bratosin *et al.* (Bratosin *et al.*, 2005) based on the use of acetoxymethyl ester calcein (Calcein-AM), a fluorescein derivative and non-fluorescent vital dye that passively crosses the membrane of viable cells and is converted by cytosolic esterases into calcein that produces intense green (530 nm) signal, and is retained by cells with intact plasma membranes. From dying or damaged cells with compromised membrane integrity unhydrolysed substrat and their fluorescent products are rapidly extruded from cells.

The membrane-permeable dye Calcein-AM was prepared as a stock solution of 10 mM in dimethylsulfoxide stored at -20°C and as a working solution of 100 µm in PBS buffer pH 7.4. RBCs ($4 x10^5$ in 200 µl PBS buffer, pH 7.4) were incubated with 10 µl Calcein-AM working solution (final concentration in Calcein-AM: 5 µm) for 45 min at 37°C in the dark and then diluted in 0.5 ml of PBS buffer for immediate flow cytometric analysis of Calcein fluorescence retention in cells. Experiments were performed at least three times with three replicates each time.

Flow cytometric analysis of phosphatidylserine exposure

To 10 µl of the RBCs suspension (10^7 cells per ml) in PBS buffer, pH 7.4, were added 90 µl of binding HEPES buffer, pH 7.4 and 5µl (0.05 µg) of FITC-Annexin V solution. After incubation for 15 min at room temperature in the dark, 400 µl of HEPES (N-(2-hydroxymethyl)piperazine-N'-(2-ethane)sulfonic acid) buffer, pH 7.4 were added and the suspension was analysed in the flow cytometer and gated for biparametric histograms FL1 (FITC fluorescence) versus FL2 (RBC autofluorescence). Ten thousand fluorescent particles of each gated population were analyzed. Data were collected on a Becton Dickinson FACScan cytometer and analyzed using CellQuestPro software. The light scatter channels were set on linear gains and the fluorescence channels on a logarithmic scale. Experiments were performed at least three times with three replicates each time.

CaspGLOW™ fluorescein active caspase-3 or -8 flow cytometric analyses

10^6 RBCs were first incubated for 1h with 1 µl of FITC-IETD-fmk for caspase-8 activity or FITC-DEVD-fmk for caspase-3 activity at 37°C under 5% CO_2 atmosphere. After 3 treatments with 0.5 ml of washing buffer, the cells were gated for parametric histograms FL1 (FITC fluorescence of CaspGLOW™). Experiments were carried out in triplicate.

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

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by the critical point method and sputter-coating with gold, samples were examined on a 35 CF JEOL SEM.

RESULTS AND DISCUSSIONS

Light scattering properties of red blood cells in Polycythemia vera

As shown in Figures 1 and 2, flow cytometric analyses announce significant morphological changes of red blood cells in Polycythemia vera. In fact, as demonstrated in Figure 1 and 2, the XGeo Mean values (cell side scatter) vary from 260 (Patient PV) compared to 299.63 (M), the statistical value for normal RBCs being 310 ± 25. In the same way, the YGeo Mean values (cell density scatter) vary from 196.87 (Patient PV) to 256.35 (M-To), the statistical value of normal RBCs being 298± 30. These morphological changes diminishes after treatment as shown in Figures 2, PVt, where the YGeo Mean increase to 222.58, value closer to YGeo Mean of normal erythrocytes (M).

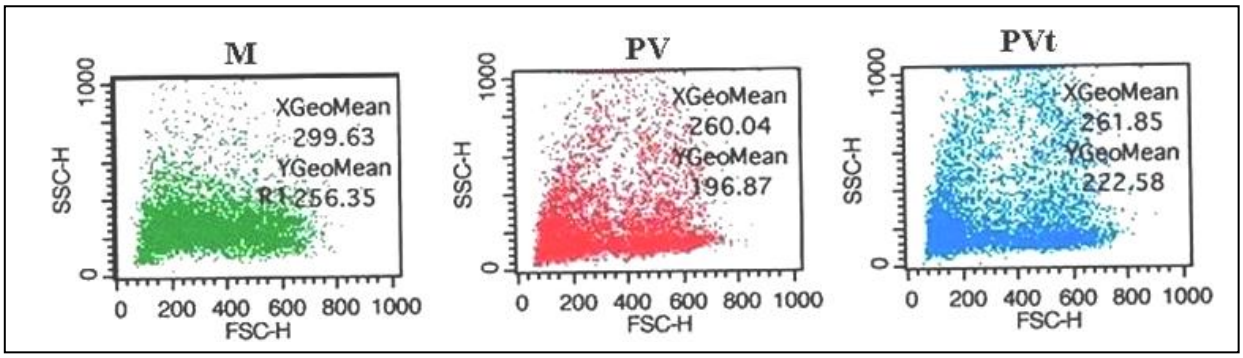

Fig. 1. Comparative dot-plot analysis FSC/SSC of morphological changes of red blood cells in Polycythemia vera. **(M):** control erythrocytes; **(PV):** red blood cells in Polycythemia vera before and after treatment **(PVt)**; Abscissae: forward scatter (cell size); ordinates: side scatter (cell density, granularity and refractiveness). Number of counted cells: 10,000. Results presented are from one representative experiment of three performed

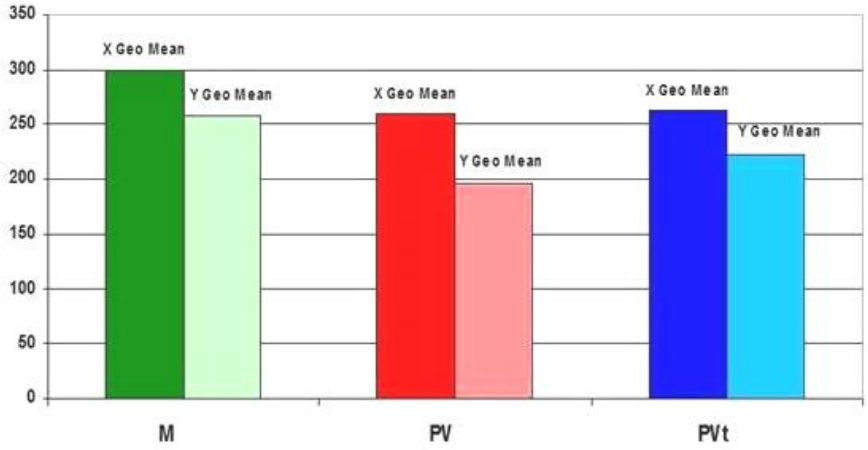

Fig. 2. Comparative histogram of X- and YGeoMean values of normal **(M)** and Polycythemia vera erythrocytes before **(PV)** and after treatment **(PVt)**. Values refer to dot-plot analyses of Figure 1

Scanning electron microscopy analysis

Scanning electron microscopy entirely confirmed these data and led to the discovery of a dysmorphic RBCs, especially stomatocytes, presented in Figure 3a to 3f, which coexist with discocytes.

Cell viability of erythrocytes

We have applied the flow cytometric assay we previously developed for the measurement of erythrocyte viability (Bratosin et al., 2005). As described in "Materials and methods", the assay is based on the use of Calcein-AM, a non-fluorescent vital dye that passively crosses the cell membrane of viable cells and is converted by cytosolic esterases into green fluorescent calcein which is retained only by cells with intact membranes. The results we obtained, illustrated by the Figure 4, reveal in RBCs of Polycythemia vera patient (PV), an Calcein means of fluorescence intensity of 160, almost double compared to MFI of normal RBCs (M). After treatment, MFI slightly decreases to 156 (PVt).

This increased esterase activity in erythrocytes frompatients with Polycythemia vera could be a possible cause of a longer life-time, which would lead to an increase in circulating hematocrit.

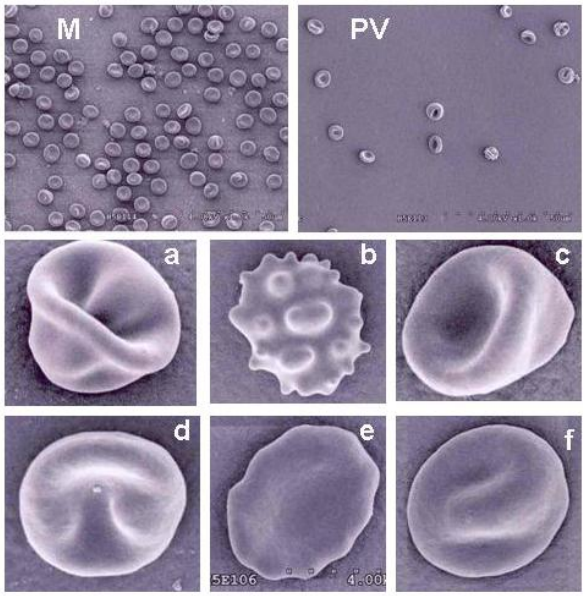

Fig. 3 Comparative scanning electron microscopy (SEM) of normal (M) and Polycythemia vera red blood cells (PV). Details of some dysmorphic Polycythemia vera erythrocytes (a to f)

Measurement of glycoconjugate sialylation

Classically, the sialic acid residues which are situated in terminal position of the glycan moieties of membrane glycoconjugates are considered as antirecognition signals for phagocytic cells. Their removal by sialidases, by demasking the penultimate β-galactosyl residues of glycans induces the capture of cells mediated by a specific lectin present in the macrophage membrane. As demonstrated by Figure 5, analysis by cytofluorimetry of the binding of FITC-labeled lectins specific for sialic acid and β-galactosyl residues shows that the means of fluorescence intensity (MFI) for *Maackia amurensis* agglutinin MAA specify for α-1,3-linked sialic acids is much smaller (23.05) compared to MFI for normal RBCs (47.88). After treatment (PVt), the MFI increase to 36.77 value.

This desialilation is also evidenced by Wheat germ agglutinin (WGA) that binds to N-acetylglucosamine, but also can interact with some glycoproteins via sialic acid residues. *Sambucus nigra* agglutinin (SNA), specific for α-1,6-linked sialic acids also show a discrete desialilation.

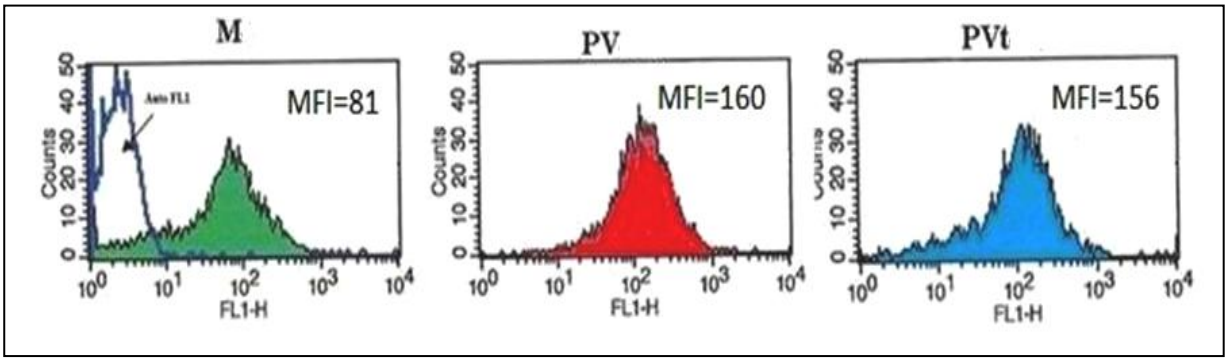

Fig. 4. Comparative flow cytometric histogram analysis of erythrocytes viability determined by cell esterase activity measurement using Calcein-AM. Human normal red blood cells **(M),** red blood cells in Polycythaemia vera before **(PV)** and after treatment **(PVt)**. Numbers represent fluorescence mean values (MFI). Abscissae: log scale green fluorescence intensity of Calcein (FL1). Ordinates: relative cell number. Number of counted cells: 10,000. Results presented are from one representative experiment of three performed.

In the same way, as demonstrated by Figure 5, binding of *Ricinus comMunis* agglutinin (RCA_{120}), specific for β-galactosyl residues, has not been fixed more on Polycythemia vera red blood cells (MFI=61.7) compared to normal erythrocytes (76.73) or after treatment, when binding is also lower (46.31), phenomenon that remains an enigma.

Study of death by annexin-V-FITC labelling

Study of death by Annexin-V-FITC labeling phosphatidylserine exposure on the outer leaflet of plasma membrane is regarded as one of the signals allowing macrophages to ingest erythrocytes. In Polycythaemia vera we did not observe any difference of phosphatidylserine externalization between normal and Polycythemia vera RBCs. The value (1.48%) is in the limit of normal RBCs statistical value: 1.5 ± 1% (data not shown)

Red blood cells in Polycythemia vera express active caspases-8 and 3

We then assessed whether erythrocytes in Polycythemia vera express active caspases. In order to precise the nature of activated caspases, we used the CaspGLOW^{TM} specific cell permeable fluorogenic substrates FITC-IETD-fmk and FITC-DEVD-fmk, which are labeled inhibitors of caspases-8 and -3, respectively.

As shown in Figure 6, a significant fluorescence was detected by flow cytometry. Thus, by comparing % of cells with active caspases, can be observed a lower level of caspase-8 active in the RBCS of

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Polycythemia vera (PV) before (0.5%) and after treatment (0.58). Inexplicably, the % of red cells with active caspase-3 is higher in both RBCs of Polycythemia vera, before (PV) and after treatment (PVt), 9.88% and 9.14% respectively, compared with normal RBCs, 2.84% (M), demonstrating that the organism tries to restore the apoptotic mechanism for maintaining the normal hematocrit.

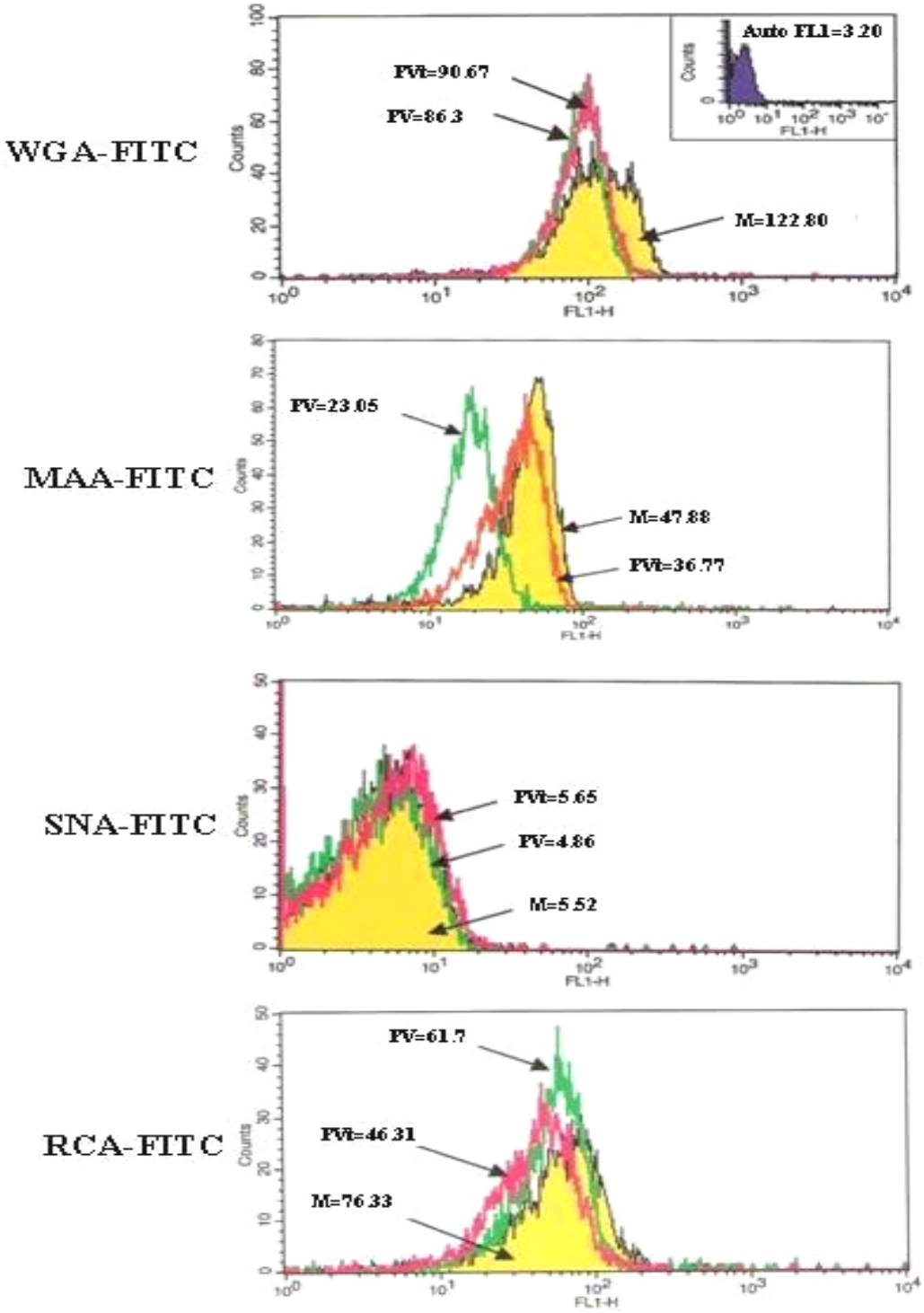

Fig. 5. Overlay (single parameter) of flow cytometric analysis of the binding of FITC-lectins specific for terminal monosaccharides: sialic acid and N-acetylglucosamine (Wheat germ agglutinin: WGA), α-1,3-linked sialic acids (*Maackia amurensis* agglutinin: MAA), α-1,6-linked sialic acids (*Sambucus nigra* agglutinin: SNA) and β-galactosyl residues (*Ricinus communis* agglutinin: RCA_{120}). MFI: means of fluorescence intensity. Number of counted cells: 10,000. Numbers represent fluorescence mean values (MFI). Data shown are from a representative experiment of three performed giving similar results.

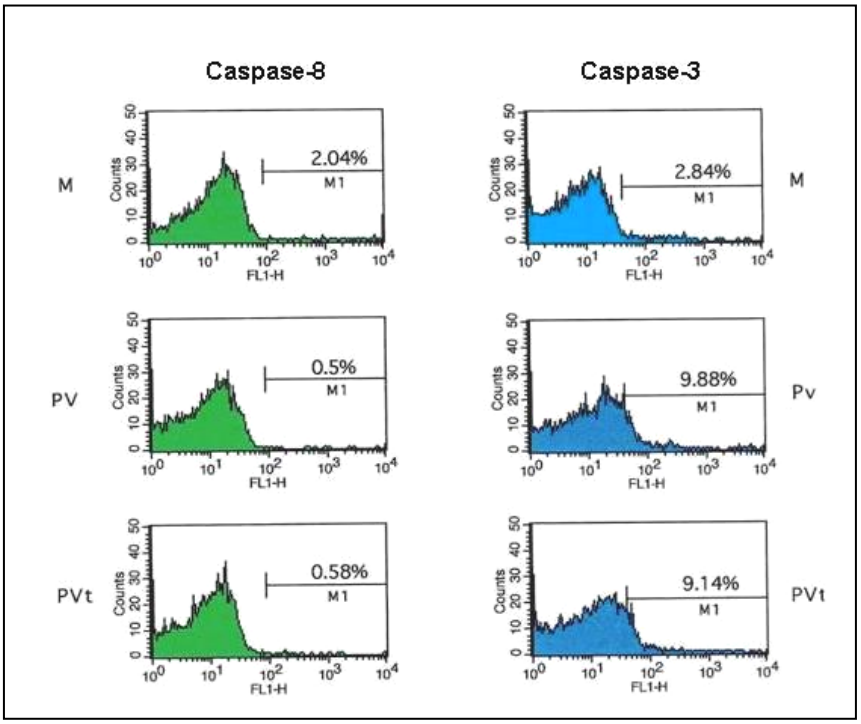

Fig. 6. Flow cytometric analysis of caspase-8 and 3 activities in RBCs of Polycythemia vera before **(PV)** and after treatment **(PVt)** compared to normal erythrocytes **(M)** using CaspGLOW^{TM}. **M1:** region of RBCs presenting actives caspases. Abscissae: log scale green fluorescence intensity of FITC-IETC-fmk for caspase-8 and of FITC-DEVD-fmk for caspase-3. Ordinates: relative cell number. Number of counted cells: 10,000. Data shown are from a representative experiment of three performed giving similar results.

Erythropoiesis is a process of red blood cells production from hematopoietic stem cells resulting from balanced proliferation, apoptosis, and differentiation that are tightly regulated by intrinsic and extrinsic signals in a differentiation stage-specific manner. The imbalance or aberrant activation of these signals has pathological consequences (Adamson *et al.*, 1976; Kralovics et *al.*, 2006; Nussenzveig *et al.*, 2007).

In a previous article (Bulai *et al.*, 2003), authors showed that the sialic acids of human erythrocyte membranes have a large diversity independent of blood groups. Indeed O-acylated-N-acetylneuraminic acids (acetylated, lactylated), O-methylated and O-sulphated were present at a significant level but N-glycolylneuraminic acid and its O-alkylated or O-acylated derivatives were not detected.

In addition, the diversity of sialic acids in the erythroleukemia RBCs membranes was extremely reduced as compared to normal cells, since only four entities instead of 10 have been characterised by their specific fragmentation mass spectra: Neu5Ac (91.5%), Neu5-Ac1,7L (7.5%), Neu4,5Ac2 and Neu4,5Ac29Lt, the two latter representing 0.5% each of the total sialic acids. This differed from membranes of normal cells in which were also present Neu5,7Ac2, Neu5,9Ac2, Neu5Ac9Lt, Neu5Ac8S and Neu (the de-N-acetylated form of Neu5Ac) and in most cases traces of Kdn. The absence of these compounds which are present in the membranes of normal cells was not due to a lack of sensitivity of the method which is able to quantify 50 times lower than those of the minor compounds present in Polycythemia vera RBCs and to detect quantities 1000 times lower. From these analyses two essential conclusions could be drawn. On the one hand, Neu5Gc was absent from Polycythemia vera RBCs and, therefore, the concept that Neu5Gc and its derivatives could be markers of malignant transformation in humans was not sustained. (Bratosin *et al.*, 2007).

CONCLUSIONS

Regarding these controversed results, we considered of interest to analyse and characterise by flow cytometry the RBCs in Polycythemia vera regarded as cancerous cells by applying the methods for characterisation of apoptotic cells, and in this study, we identified that analyses of red blood cells in Polycythemia vera by light scattered measurements and scanning microscopy identified morphological forms that deviate from classical discoid shape, showing cup-shaped or stomatocytes.

Measurement of glycoconjugate sialylation using lectines demonstrates a low degree of membrane glycoconjugates sialilation of Polycythemia vera RBCs but which is close to normal after treatment. The RBCs viability determined by Calcein-AM methods showed a high viability of Polycythemia vera RBCs

compared to normal erythrocytes and after treatment the erythrocyte viability have a light reduction. Phosphatidylserine exposure level of red blood cells in Polycythemia vera was not different from normal erythrocytes, although it is a marker of apoptosis, and increased percentage of cells with active caspase-8 and -3 before and after treatment compared to normal RBCs demonstrates that the organism tries to restore the apoptotic mechanism for maintaining the normal hematocrit.

Our observations may contribute for understanding the survival of red blood cells in Polycythemia vera and participate to elucidation of this mechanism in pathogenesis of this disease.

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