

FLOW CYTOMETRIC ASSAY FOR THE EVALUATION OF PHAGOCYTOSIS AND OXIDATIVE BURST OF POLYMORPHONUCLEAR LEUKOCYTES AND MONOCYTES IN MYELODYSPLASTIC DISORDERS

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ABSTRACT

Background. Myelodysplastic syndromes are clonal diseases characterized by pancytopenia of variable degree. Neutropenia is common and several morphologic and functional abnormalities of polymorphonuclear neutrophilic granulocytes (PMNs) and/or monocytes have been described. On the basis of these observations, the phagocytic and oxygen intermediates production of PMNs and monocytes was determined in a group of forty-seven patients affected by myelodysplastic syndromes of varying severity.

Methods. A rapid, simple and reliable flow cytometric method was developed to evaluate, in a one-step procedure, the phagocytosis rate and the oxidative burst in PMNs and monocytes using a small amount of whole blood.

Results. Phagocytosis of PMNs and monocytes was not significantly reduced in refractory anemia (RA), while in refractory anemia with excess of blasts (RAEB) and in chronic myelomonocytic leukemia (CMML) a clear decrease ($p < 0.05$) of this function was found in both PMNs and monocytes. The production of oxygen intermediates by PMNs and monocytes was significantly ($p < 0.01$) reduced in RA as well as in RAEB and in CMML.

Conclusions. This study indicates the presence in myelodysplastic syndromes of a severe reduction in phagocytosis and oxygen intermediates production (two crucial functions to protect the host against pyogenic agents) in both PMNs and monocytes. This observation could explain the severe morbidity and mortality from infections in patients affected by these hematological malignancies.

Key words: myelodysplastic syndromes, monocytes, granulocytes, phagocytosis

Myelodysplastic disorders are clonal diseases resulting from a lesion in the pluripotential stem cell pool.¹ The major specific pathophysiologic mechanism is ineffective hemopoiesis, that is, defective maturation of precursor cells and failure to accumulate adequate numbers of circulating mature cells.^{2,3} Neutropenia is common (about 50% of patients at the time of diagnosis)⁴ and morphologic⁵ and functional abnormalities of polymorphonuclear neutrophilic granulocytes (PMNs) and/or monocytes have been described.⁶ Specific

neutrophil granules can be decreased in number, producing hypogranular cells,⁷ and bactericidal capability and chemotaxis may be impaired.⁸ Since phagocytosis and the production of oxygen intermediates, referred to as *oxidative burst*,^{9,10} are the critical steps in the destruction of invading pathogenic bacteria, an assessment of these functions may be clinically important in these patients to prevent infectious complications.

Recently, we developed a rapid and sensitive method for evaluating simultaneously the

phagocytosis rate and the oxidative burst activation of PMNs with a two-color flow cytometric method.¹¹ Since isolation of PMNs from blood requires time-consuming methods and can alter phagocytic function, we developed a new procedure that employs whole blood for a simultaneous evaluation of the phagocytosis rate and oxidative burst activation. Identification of the different types of leukocytes is made possible by flow cytometry detection of differences in cell light scattering properties. In this work we present data obtained with this fast, easy and sensitive method on the phagocytosis and oxygen production of PMNs and monocytes in patients affected by myelodysplastic syndromes.

Patients

Forty-seven patients referred as outpatients to the Hematology Department of Istituto di Patologia Medica were selected for the present study. Bone marrow biopsy and aspirate were obtained from all subjects at presentation. The diagnosis was formulated according to criteria proposed by the French-American-British Group.¹² No patient had been given any therapy during the previous 3 months, and each participant was studied at least three times.

Forty healthy subjects recruited from among the medical and nursing staff of our Institute were used as controls. Two children affected by chronic granulomatous disease (CGD) were also tested to support the method.

Materials and Methods

Bacteria

Staphylococcus aureus (S.A.) strain ATCC 25923 was grown overnight at 37°C in tryptic soy broth (Difco Laboratories, U.K.) with aeration. Heat-killed microorganisms were labelled with fluorescein isothiocyanate (FITC) purchased from Sigma (St. Louis, MO, USA) according to a previously described technique.¹¹ The fluoresceinated microorganisms were washed three times in Hank's Balanced Salt Solution (HBSS), resuspended to a concentra-

tion of $7-9 \times 10^8$ /mL and stored at -20°C in small aliquots. When indicated, bacteria were opsonized by incubation with 20% normal AB sera for 30 minutes at 37°C in a shaking water bath.

Phagocytosis and reactive oxygen intermediates production

Hydroethidine (HE), purchased from Serva (Heidelberg, Germany) was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 1 mg/mL and stored at -70°C . Since HE gives a red fluorescence emission as it is rapidly oxidized to ethidium bromide (EB) in the presence of activated PMNs or monocytes, a fraction of cells was treated as controls with phorbol myristate acetate (PMA) (Sigma Chemicals Co., St. Louis, MO, USA), a potent activator of oxidative burst. A mixture of 100 mL of heparinized whole blood, 10 mL of HE (100 ng) and 20 mL of FITC-labelled S.A. suspension was placed in a plastic tube. To measure reactive oxygen intermediates (ROI) production or phagocytosis separately, 20 μL of fluoresceinated microorganisms were added to either HE-pretreated or untreated whole blood, respectively, at several bacteria-to-cell ratios in different series of tubes. After various time intervals of incubation in a shaking water bath at 37°C, each sample was lysed at room temperature with Immunoprep (Coulter Diagnostics, Hialeah, FL, USA) or with 2 mL of Lysing solution (Becton Dickinson, San José, CA, USA) according to the procedures suggested by the manufacturer. After centrifugation, the pellet was resuspended in 1 mL PBS-EDTA 0.1 M and immediately analyzed.

Flow cytometry

A FACScan (Becton Dickinson Immunocytometry Systems, San José, CA, USA) was used to detect both FITC and HE fluorescence. Fluorescence parameters from single cells were collected using a logarithmic amplifier after gating on the combination of forward and perpendicular light scatter. Red fluorescence from EB was collected through an FL2 channel with a DF 655/35 or 585/42 nm filter; and green fluorescence from FITC through an FL1 channel

with a 530 nm band pass filter; 10,000 cells were analyzed per tube and data were acquired in the list mode and processed using Consort 32 Lysis II software. The fluorescence distribution was displayed as a single histogram or two-color contour plot analysis, and the percentage of fluorescent cells was determined. The events were acquired with the linear mode for forward and side scattering and with the logarithmic mode for FL1 and FL2. Compensation of FL1 percent FL2 and FL2 percent FL1 ranged between 3-6% and 20-35%, respectively.

Histology

Marrow smears were stained according to standard procedures. Bone biopsies were placed in B5 solution and, 2 hours later, in ethanol 70%. All samples were examined by the same experienced pathologist following standard criteria.

Statistical analysis

Data are expressed as mean \pm SD. Statistical analysis was performed by using the statistical package SPSS. For continuous variables an analysis of variance between groups was calculated by ANOVA (one-way), where *p* represents the probability of Snedecor's F that the means of interval-dependent variables of the two groups are equal. Categorical variables were analyzed with a Pearson χ^2 .

Results

Phagocytosis and reactive oxygen production

Figure 1 shows an example of flow cytometric analysis. In Figure 1A the distribution of particles is displayed according to their forward- and side-scattering properties: regions R1 and R2 are set to select PMNs and monocytes, respectively. In setting gates, the increase in granularity and the subsequent modifications in forward- and side-scattering of PMNs following phagocytosis (or PMA stimulation) are indicated in Figure 1B. In the following figures the effects of phagocytosis and/or oxidative burst activation are shown: on the ordinates the red

fluorescence (due to HE oxidation), and on the abscissae the green fluorescence (due to bacteria ingestion). In Figure 1C a contour plot of unstimulated PMNs is shown. Figure 1D displays the green fluorescence emission due to PMNs that ingested FITC-labelled S.A., and Figure 1E depicts the red fluorescence emission due to ROI production and subsequent HE oxidation to EB within phagocytic cells after PMA stimulation. Figure 1F illustrates the simultaneous evaluation of S.A. phagocytosis and oxidative product formation: the contour plot shows the phagocytic cells displaying green and red fluorescence simultaneously. This event can be quantified as percent of fluorescent cells versus total counted.

The rate of phagocytosis and ROI production was studied at different cell-to-bacteria ratios. The percentage of double fluorescent PMNs increases by increasing the number of bacteria added to the whole blood system. A maximum effect was obtained when a ratio of 20:1 microorganisms to cells was employed.

Control group

The mean age of the control group was 32 \pm 9 years. The tests were performed with a ratio of 20:1 S.A. to cells over a 15-min incubation time. The mean value of the phagocytosis rate, expressed as percentage of cells displaying green fluorescence, of the PMNs (91.66 \pm 5.75) was significantly ($p<0.05$) higher than that of the monocytes (83.5 \pm 10.1). The oxygen intermediates production, expressed as percentage of cells displaying red fluorescence, was also significantly ($p<0.01$) higher in PMNs (91.6 \pm 5.7) than in monocytes (70.0 \pm 12.7).

Patients

The mean age of the patients was 59 \pm 18 (range: 33 to 89 years). In the subjects affected by myelodysplastic syndrome, marrow examination showed the presence of refractory anemia (RA) in 31 cases, refractory anemia with excess of blasts (RAEB) in 14 cases (four in transformation) and two with chronic myelomonocytic leukemia (CMML). Five patients (10.6%) showed slight anemia, seven (14.9%) moderate and two (4.2%) severe anemia (Hb

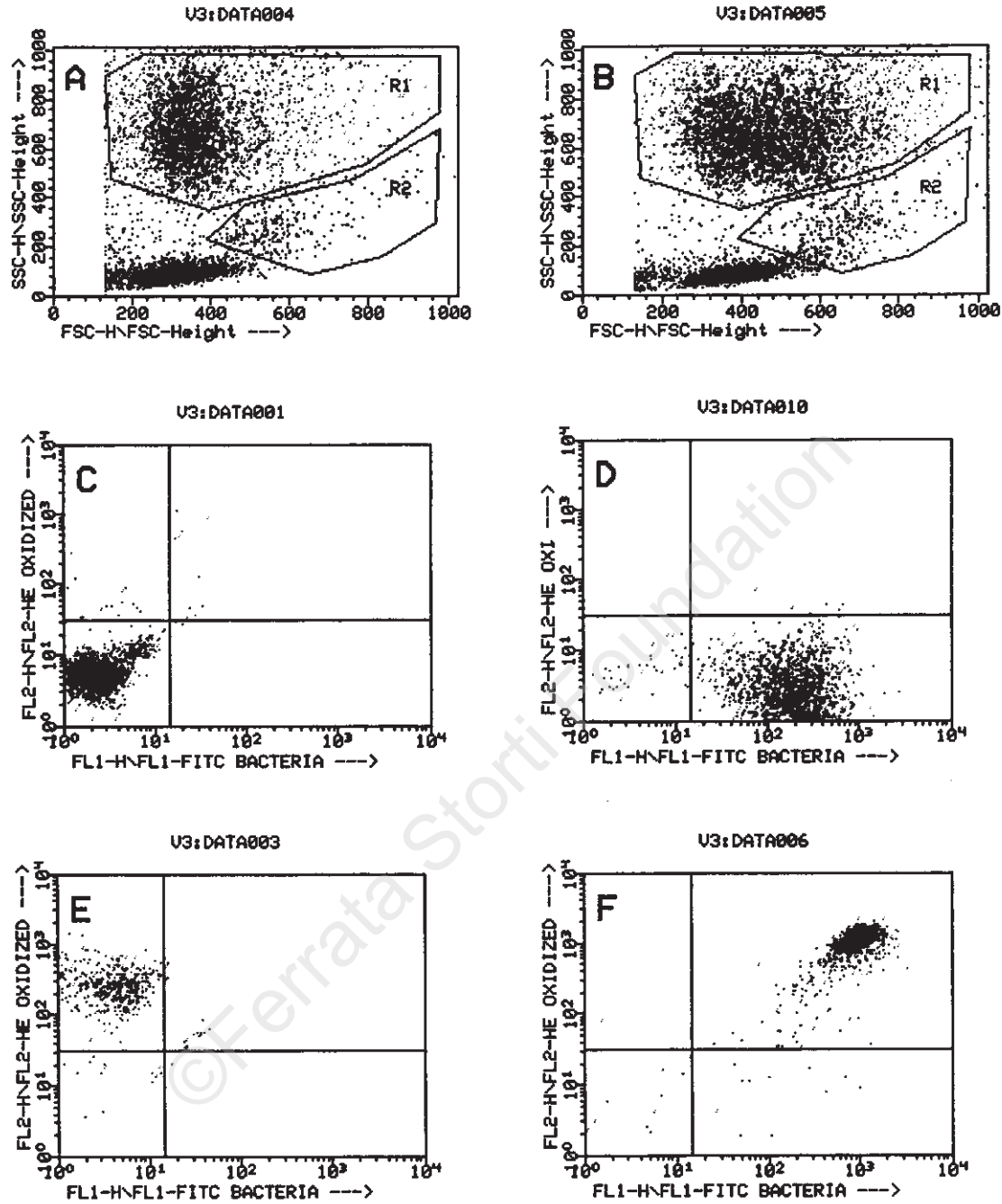


Figure 1. Top panels: forward- and side-scattering dot plot of whole blood without (A) and with phorbol myristate acetate (B); regions R1 and R2 were set for polymorphonuclear neutrophilic granulocytes (PMNs) and monocytes, respectively. Bottom panels represent the FL1 (green) and FL2 (red) contour plots of unstimulated PMNs (C). PMNs that ingested FITC-labelled bacteria (D). PMNs producing ROI when stimulated with PMA (E), and PMNs producing ROI during phagocytosis of labelled bacteria (F). See text for details.

less than 8.0 g/dL). Granulocytopenia was common: the granulocyte count was less than $1.5 \times 10^9/L$ in twenty-eight subjects (59.6%).

Thrombocytopenia was present in nine (19.1%) subjects, but only one of them showed a hemorrhagic syndrome. Five patients needed

Table 1. Hematological and biochemical characteristics of the patients.

Parameters	normal values	RA (31 cases)	RAEB (14 cases)	CMML (2 cases)
Age		67±10	71±6	65±3
Sex (M/F)		10/19	6/3	1/1
Hb (g/dL)	12-16	11.9±2.5	11.0±2.3	13.1±0.2
Neutrophils (x10 ⁹ /L)	2-7	1.7-0.9	1.3-0.7	3.25±0.2
Monocytes (x10 ⁹ /L)	0-1	0.28±0.2	0.34±0.2	5.0±0.4
Platelets (x10 ⁹ /L)	130-400	210-80	171±93	68±41
ALT (U/L)	5-40	36±9	78±15	12±14
AST (U/L)	5-40	23±10	50±66	18±12
ALP (U/L)	120-220	139±65	97.0±28.9	79±29
Creatininemia (mg/dL)	<1.3	1.1±0.4	0.9±0.2	0.8±0.7

RA: refractory anemia; RAEB: refractory anemia with excess of blasts; CMML: chronic myelomonocytic leukemia.

blood transfusions during the follow-up, with blood requirements ranging from one to six units per month. The main laboratory characteristics of the patients are summarized in Table 1. Two subjects affected by RAEB also showed hepatitis C virus infection, and this explains the high mean ALT and AST values in this group of patients.

In the myelodysplastic syndromes, phagocytosis and ROI production were reduced in both PMNs and monocytes. In RA, phagocytosis of PMNs and monocytes did not differ from controls, while in RAEB(t) and CMML a significant ($p<0.05$) decrease was found. The ROI production was significantly ($p<0.005$) impaired in monocytes as well as in PMNs in both RAEB(t) and CMML, while a slight decrease ($p<0.05$) in this function was observed in RA. The means of phagocytosis and ROI production in the different diseases are indicated in Tables 2 and 3.

In the two patients affected by CGD, a normal phagocytosis rate was obtained (87% and

83% of PMNs and monocytes, respectively, developed green fluorescence), but after ingesting bacteria, neither type of cell was able to produce ROI (0.5%).

Discussion

Lately, an increasing number of studies have reported on flow cytometric evaluation of PMN and monocyte functions, which are of clinical relevance for the diagnosis of CGD and are also useful for studying the interactions between bacteria and PMNs in healthy subjects as well as in immunocompromised hosts.¹³⁻¹⁶ We recently published a flow cytometric method for simultaneous evaluation of PMN phagocytosis rate and oxidative burst.¹¹ In the present work that technique has been simplified by employing only small amounts of whole blood. In fact, assessment of phagocytosis and ROI functions on purified cells has several disadvantages, such as the need for large blood volumes and long

Table 2. Phagocytosis rate in controls and in patients affected by myelodysplastic syndromes.

Subjects	Neutrophils	Monocytes
Control	94.9±5.5	83.5±10.1
RA	95.1±7.1	80.1±11.9
RAEBt	84.4±13.1*	61.9±24.2*
CMML	82.5±3.5*	51.4±1.4°

Data are expressed as percentage of cells displaying green fluorescence. RA: refractory anemia; RAEB: refractory anemia with excess of blasts (t: in leukemic transformation); CMML: chronic myelomonocytic leukemia. * $p<0.05$; ° $p<0.01$.

Table 3. Oxygen intermediate production in controls and in patients affected by myelodysplastic syndromes.

Subjects	Neutrophils	Monocytes
Control	91.6±5.7	70.0±12.7
RA	87.5±10.1*	57.9±19.8*
RAEBt	72.2±18.4°	51.3±23.6°
CMML	71.1±1.4°	49.5±13.5°

Data are expressed as percentage of cells displaying red fluorescence. RA: refractory anemia; RAEB: refractory anemia with excess of blasts (t: in leukemic transformation); CMML: chronic myelomonocytic leukemia. * $p<0.05$; ° $p<0.005$.

intervals between blood collection and functional determinations. The present method allows simultaneous measurement of bacterial ingestion and ROI production in a short time and with a small amount of whole blood. Moreover, the use of some common lysing solutions like FACS Lysing Solution (Immunocytometry Systems, Becton Dickinson, USA) or Immunoprep (Coulter Diagnostics, USA) that contain paraformaldehyde as fixative allows a distinction to be made between adherent and ingested bacteria, avoiding further steps with vital dyes as quenching agents.¹⁷ Another advantage of the present method is the possibility of determining monocyte functions, thus eliminating the laborious steps needed for purification of these cells.^{18,19} Moreover, no pre-opsonization of bacteria is necessary because the incubation time in whole blood ensures sufficient opsonization of microorganisms in a physiological environment.²⁰

Study of phagocytosis and ROI production in myelodysplastic syndromes indicates that these functions are significantly reduced in both PMNs and monocytes. However, impairment of these two functions is not homogeneous and the two do not exhibit parallel behavior. Phagocytosis of PMNs is more conserved than ROI production; in fact, no significant difference was found between controls and patients affected by RA, while in RAEB(t) and CMML this function is significantly ($p < 0.05$) reduced. Conversely, ROI activity also shows a statistically significant ($p < 0.05$) decrease in RA, while in both RAEB(t) and CMML this defect is much more pronounced (~22%, $p < 0.005$).

Phagocytic function also seems to be more conserved than ROI production in monocytes. However, in these cells the two activities show a high degree of variability, even in normal subjects. This may be due to the functional heterogeneity of monocytes or to the difficulty in separating these cells from large lymphocytes. The functional defects of monocytes are much more pronounced in CMML than in the other myelodysplastic syndromes, but this is not surprising since the monocyte population consists of neoplastic cells.

The impairment of phagocytic activity and

ROI production is not correlated with the absolute PMN or monocyte count in the peripheral blood. In fact, linear regression analysis between the PMN and monocyte counts and phagocytosis and ROI activity did not show any correlation (r ranging from 0.11 to 0.21). Moreover, the granulocyte and monocyte counts showed no significant differences between RA and RAEB(t). This indicates that the functional defects are independent of peripheral blood leukocyte count and that the metabolic abnormalities may be present even in subjects with an almost normal leukocyte count. Several mechanisms could determine defective ROI production in myelodysplastic syndromes, such as: a) a reduced number of (or structurally altered) surface adhesion receptor molecules (selectins or integrins),^{21,22} which, when they interact with an antigen, contribute to the development of cell function (e.g. release of cytolytic molecules, ROI production and destruction of target cell membrane);^{23,24} b) defects in the membrane-bound or cytosolic components of NADPH-oxidase, which lead to reduced activity of the enzyme (as occurs in CGD).^{8,25}

In a fraction of our patients we also determined the concentration of the CD11/CD18 complex on the cell surface of PMNs and monocytes, and the number of these integrins seemed to be significantly reduced in both cells; the decrease in CD11/CD18 correlates well ($r: 0.75$) with the reduction in ROI production (unpublished observations). This may indicate that adhesion molecules may be involved in the functional defects of PMNs and monocytes in myelodysplastic syndromes. Integrins are useful not only for ROI production and bacteria destruction, but also for interaction between T-cells and monocytes during the early stage of immune responsiveness. Since this mechanism seems to be partially defective, reduced phagocytosis and ROI production, associated with an impaired immune system, could explain the high frequency of infections in these patients. Although other PMN functions such as motility, chemotaxis and non-oxidative bactericidal mechanisms (lipases, proteases, glycosidases and other defensins) were not considered in present study, the defense against pyogenic bacteria is

certainly defective. These alterations, alone or in combination with other mechanisms, may be responsible for the high mortality from infective agents in these subjects.

In conclusion, this work indicates that the present flow cytometric method is an easy, reliable and time-saving tool for obtaining useful information about the phagocytosis and ROI production of PMNs and monocytes. In the myelodysplastic syndromes impairment of these two functions is present in both PMNs and monocytes, especially in more advanced diseases, explaining the high morbidity and mortality from infections in these patients.²⁶

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