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Brief definitive report

Volume, conductivity, and scatter changes of activated polymorphonuclear leukocytes: an estimation by Coulter Counter STKS analyzer

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Summary. Suspensions of phorbol myristate acetate – activated polymorphonuclear leukocytes were analyzed with a Coulter Counter STKS hematological analyzer. Phorbol myristate acetate activation induced an increase in polymorphonuclear leukocyte volume and conductivity, while scatter was unchanged. Phorbol myristate acetate-activated neutrophils in a suspension containing nitroblue tetrazolium showed increased scatter. The rise in scatter was phorbol myristate acetate dose dependent, completely inhibited by diphenylene iodonium and partially by dimethyl sulfoxide, two inhibitors of NADPH oxidase. Zymosan-activated polymorphonuclear leukocytes were notably larger with a characteristic position on discriminant function 1 display (volume versus scatter) of the analyzer. Volume and conductivity changes were seemingly inexplicable features of phorbol myristate acetate activation. The rise in scatter was produced by cytoplasmic precipitation of reduced nitroblue tetrazolium and thus by O_2-generation in phorbol myristate acetate-activated neutrophils. Zymosan phagocytosis was responsible for the notable rise in polymorphonuclear leukocyte volume. The analysis of activated polymorphonuclear leukocytes by Coulter Counter STKS may provide useful information on their activation and a pragmatic approach for studying function.

Key words: Coulter Counter STKS – Activated neutrophils

Introduction

Coulter Counter STKS (Coulter, Hialeah, Fla.) is a hematological analyzer that provides leukocyte differential counting using three measurements: individual cell volume, high-frequency conductivity, and laser light scatter. A suspension of leukocytes is passed through a small orifice at the same time as an electric current. The individual blood cells passing through the orifice introduce an impedance change in the orifice determined by the size of the cells. If a high-frequency current is applied to the current field, the current penetrates the surfaces of the particle to reveal internal composition. The angle of the scatter light depends on particle size and refractability. A two-dimensional scatterplot, discriminant function 1 (DF1), derives primarily from light scatter (x-axis); the y-axis represents volume. Single-parameter histograms of volume, conductivity, and light scatter are also available for data interpretation. If a pure suspension of polymorphonuclear leukocytes (PMN) is analyzed with Coulter STKS the instrument provides a specific estimation of PMN volume, conductivity, and scatter. In this paper we report some physicochemical changes of in vitro activated human PMN, as these changes are shown and expressed by Coulter STKS.

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Materials and methods

Reagents. Ficoll (type 400) 1077 and 1119 containing sodium diatrizoate (Histopaque 1077 and 1119), phorbol myristate acetate (PMA), zymosan A, nitroblue tetrazolium (NBT), dimethyl sulfoxide (DMSO), and phosphate-buffered saline (PBS) were purchased from Sigma (St. Louis, Mo.). Diphenylene iodonium (DPI) was a generous gift from Dr. A. R. Cross (University of Bristol, UK). A PMA solution was made in DMSO at a concentration of 5 mg/ml and stored in aliquots at –20 °C. Immediately before use, PMA was diluted with PBS to the desired concentration. Zymosan was prepared for use by boiling in PBS (10 mg/ml) for 10 min, washing twice with 0.15 M TRIS-buffered saline, pH 7.4, and then resuspending in PBS. DPI was dissolved in ethanol (1 mM) before being diluted in PBS. A DMSO solution in PBS was prepared just before use.

PMN separation. Aliquots of venous whole blood (6 ml) with potassium EDTA as anticoagulant (1.5 mg/ml) were layered on two discontinuous gradients each composed of 3 ml Histopaque 1119 and 3 ml Histopaque 1077. The two gradients were centrifuged at 700 g for 30 min at 20 °C in a swinging-bucket rotor. Lymphocytes, monocytes, and platelets were withdrawn and discarded from the serum-Histopaque 1077. Neutrophils at the Histopaque 1077 and 1119 interface were collected, immediately diluted with PBS, cen-

trifuged, washed three times with PBS, counted, and resuspended with PBS at PMN concentrations not lower than 3×10^6 cells/ml. This procedure allowed the study of populations that were $98 \pm 2\%$ neutrophils with few contaminating erythrocytes. PMN viability was 98%–100% as assessed by the trypan blue exclusion test.

PMN activation and inhibition studies. Aliquots of PMN suspension (0.25 ml) were mixed with PBS (0.20 ml) or NBT (5 mM) in PBS in siliconized glass tubes (5×0.7 cm) containing a Teflon stir bar revolving at 2000 rpm at 37 °C in the holder of a common aggregometer. After a 5-min delay to allow warming of suspensions, an aliquot (50 µl) of the solution containing the inhibitor, or PBS alone (controls), was added. Inhibitors of PMA activation and their final concentrations were as follows: DMSO 300 mM, DPI 1 and 10 µM. After a further 10-min incubation, PMN activation was started by adding PMA solution (50 µl) ± at 100 ng/ml final concentration. In some experiments, 10, 25, and 50 ng/ml the final concentrations of PMA were used. Analysis of the Coulter STKS was performed after a 15-min incubation by using 100-µl aliquots of each suspension. The effects of inhibitors on PMN viability were estimated by the trypan blue exclusion test.

PMN activation by zymosan was carried out by adding to prewarmed suspensions (0.25 ml) the same volume of PBS containing fresh autologous serum (20%) and Zymosan (1 mg/ml). Analyses on Coulter STKS and STKR were performed after a 30-min incubation with the stirring device described above. STKR, another hematological Coulter analyzer, provides a histogram of volume distribution with precise cell sizing (fl).

Smears of PMN suspensions without NBT were stained with May-Grünwald Giemsa and with hydrogen peroxide/4-chloro-1-napthol for myeloperoxidase (MPO) activity [7]. NBT reduction to the formazan salt was also estimated microscopically using smears from PMN suspensions containing NBT.

Results

PMA-activated neutrophils were shown by Coulter STKS to be larger and to have a higher conductivity than resting neutrophils (Fig. 1). The histograms of volume and conductivity demonstrated a high frequency (92% of cases) of a double population, indicating that a percentage of neutrophils were hyper-responsive to PMA with respect to volume conductivity. Scatters were invariably unchanged. The microscopic evaluation of PMA-activated PMN demonstrated little cytoplasmic affinity for May-Grünwald stain and decreased MPO activity. MPO-positive granulations were scantier in the largest neutrophils, indicating the coexistence of a double PMN population where volumes and MPO-positive granularity were inversely correlated.

Activation of PMN suspensions by PMA in the presence of NBT produced a strong cytoplasmic precipitation of formazan, microscopically detectable as dark needle-shaped granulations. Coulter STKS histograms of these formazan-containing neutrophils showed increased scatter when compared with resting neutrophils (Fig. 2a). The shift to the right of PMA scattering in the presence of NBT demonstrated a PMA dose dependence at PMA concentrations of 0–50 ng/ml (Fig. 2b). Figure 2 also shows that in activated PMN two peaks of increase of scattering were present, one of which (that corresponding to the maximum scatter increase) was smaller, but present in all the assays. This is in agreement with the existence of two populations of PMN, different in the extent of their activation by PMA. Microscopic semiquantitative evaluation of cytoplasmic formazan confirmed the PMA dose-dependent reduction of NBT and the existence of two populations of PMN showing a high and medium amount of formazan deposits.

The possibility of detecting phagocytosis of opsonized zymosan with Coulter STKS was also explored (Fig. 3a, b). Zymosan-activated neutrophils looked markedly enlarged, nearly all of these being outside the upper limit of DF1 (Fig. 3b). The volume increase was due to phagocytosis and not to an effect of serum; indeed volumes of resting PMN were unchanged if the cells were incubated in autologous serum that had been pretreated for 30 min with 1 mg/ml zymosan and then centrifuged for 10 min at 700 *g* to remove the zymosan particles. The position of zymosan-activated neutrophils on the DF1 display of the Coulter STKS is also characteristic of cell aggregates, but this was excluded by microscopic examination of stained smears and by the fact that resting and activated PMN counts were unchanged. Microscopic examination also confirmed that phagocytosing PMN are much larger

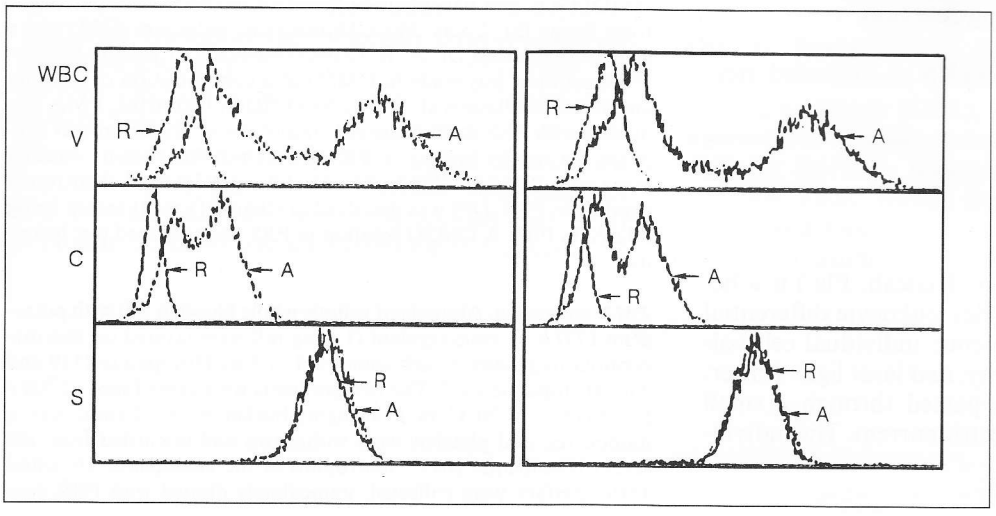

Fig. 1. STKS histograms of volume (*V*), conductivity (*C*) and scatter (*S*) of resting (*R*) and phorbol myristate acetate (PMA)-activated (*A*) neutrophils (two representative cases). After a 5-min preincubation in phosphate-buffered saline, polymorphonuclear leukocytes (PMN) were incubated for 15 min in the absence (*R*) or in the presence (*A*) of 100 ng/ml PMA

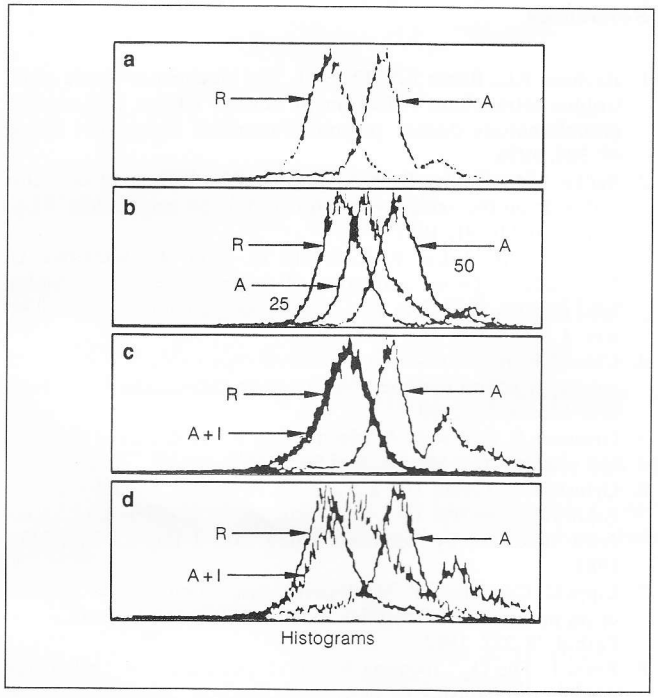

Fig. 2a–d. STKS scatter histograms of resting (*R*) and PMA-activated (*A*) neutrophils in suspensions containing nitroblue tetrazolium, without or with inhibitors (*A* + *I*). For inhibition, PMN suspensions were preincubated with inhibitor 10 min before PMA. **a** PMA 100 ng/ml; **b** PMA 25 and 50 ng/ml; **c** PMA 100 ng/ml, diphenylene iodonium 10 μM; **d** PMA 100 ng/ml, dimethyl sulfoxide 300 mM

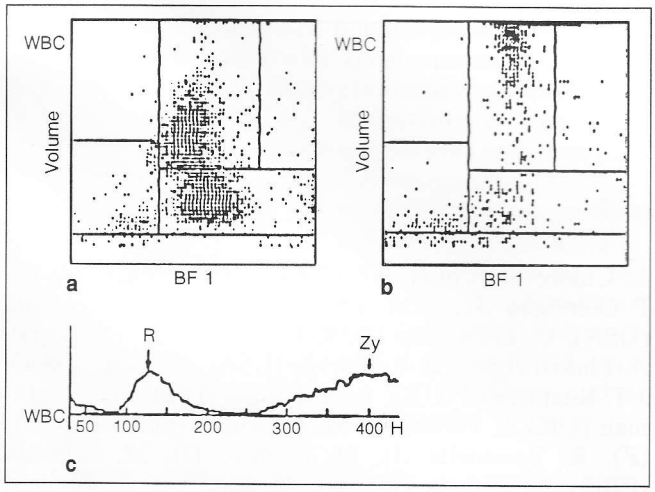

Fig. 3a–c. Volume changes of resting and zymosan-activated neutrophils. **A, B** Distribution of PMN volumes on STKS; **A** resting cells, **B** cells incubated 30 min with 10% autologous serum and 0.5 mg/ml zymosan. STKS PMN counts were 6.3×10^6 ml and 6.2×10^6 ml in **A** and **B** respectively. **C** Distribution of volumes of resting (*R*) and Zymosan activated (*Zy*) PMN as assessed on STKR

than resting cells, containing a mean of five to seven particles per cell (data not shown). Volume measurements on Coulter STKR demonstrated a population of activated PMN with volumes greater than 250 fl, while the volume of resting cells was always less than 250 fl (Fig. 3c). The volume of PMN that phagocytosed zymosan was about twice the volume of resting cells.

The rise in scatter produced by PMA-activated neutrophils in suspensions containing NBT was fully inhibited by the NADPH oxidase inhibitor DPI and partially by DMSO (Fig. 2c, d). Microscopic examination of smears demonstrated no formazan production by DPI-treated neutrophils and only a small NBT reduction in DMSO-treated PMN. Cell viability was unaffected by these inhibitors.

Discussion

STKS measurements of resting and activated neutrophils assessed: (1) the increased volume of either zymosan- or PMA-activated neutrophils, (2) the increased conductivity of PMA-stimulated neutrophils, (3) the diphasic response to PMA of increased volume and conductivity with a double PMN population, one of which was more sensitive to PMA, and (4) the increase in scatter when PMA activation was carried out in PMN suspensions containing NBT. The increased volume of zymosan-activated neutrophils was not unexpected, since they depend on zymosan phagocytosis, and therefore on a physical change. The increased volume of PMA-activated neutrophils is open to various interpretations on physicochemical and metabolic grounds. We excluded a physical action of PMA on neutrophils since the osmolality of the PMN suspension, either with or without PMA, was 234 mosmol/kg. The PMA-dependent volume increase could be caused either by formation of internal vacuoles, due to activation of endocytosis and/or by water influx as a consequence of activation of Na^+/H^+ exchange stimulated by protein kinase C [5]; a similar volume increase has been described also in platelets [6].

Since conductivity reflects the internal composition of the cell, the increased conductivity of PMA-activated neutrophils might be associated with degranulation, as shown microscopically by the cytoplasmic loss of affinity for the May-Grünwald stain and rarefaction of MPO-positive granules.

PMA activation affects the increase in scatter when neutrophils are incubated in suspensions containing NBT. This physical change might be explained as a cytoplasmic precipitation of formazan salt. It is known that NBT reduction in phagocytes occurs by a chemical reaction between the dye and the O_2^- generated during the burst [1]. The scatter increase may be the evidence of NBT reduction by O_2^- production in response to PMA, in a dose-dependent fashion, from 50 to 10 ng/ml. The further proof that scatter changes PMA-activated neutrophils were induced by O_2^- production and NBT reduction was provided using two inhibitors of O_2^- generation. DPI is a potent specific inhibitor of the oxidase system; the site of action is known to be at the level of, or prior to, the flavoprotein since it prevents reduction of both the cytochrome *b*-245 and flavin in steady-state experiments [4]. DMSO (300 mM) significantly suppressed the production of O_2^-, hydrogen peroxide, and hypochlorous acid by human neutrophils stimulated with

either PMA or opsonized zymosan. The inhibitory effect of DMSO on the production of O_2^- and hydrogen peroxide suggests that this compound might directly affect the membrane-associated NADPH oxidase [2]. The inhibitors do not affect volume and conductivity changes induced by PMA, indicating that these events are not directly linked to activation of NADPH oxidase.

It is known that PMN stimulation by PMA induces the activation of protein kinase C and, owing to this, the phosphorylation of proteins and activation of NADPH oxidase [3, 9]. The first phase of NADPH oxidase activation corresponds to the interaction between the stimulus and the cell surface (recognition). The second phase induces molecular and functional modifications of the plasma membrane and intracellular consistuents (transduction) [8]. The physical changes seen with Coulter STKS in PMA-treated neutrophils may be features of the transduction process, unaffected by inhibitors of NADPH oxidase.

If volume and conductivity changes are indexes of various biological events during PMN activation, their measurement by STKS may provide further information in addition to the semiquantitative assay of O_2^- generation. Coulter STKS, a widely available hematological analyzer, may help in the study of PMN function and in the diagnosis of various genetic and acquired defects of PMN that may affect these cells at different biochemical levels.

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Announcement

Advanced course on "Cell Differentiation and Death"

Ettore Majorana Centre for Scientific Culture, International School of Medical Sciences, Pediatric Hematology and Oncology, Erice, Sicily. 6–11 May, 1992. *Directors of the course:* G. P. Tonini (I), C. M. Croce (USA), G. Melino (I), and L. Massimo (I). The International School of Medical Sciences in Erice announces that a course on the different biological and molecular aspects of cell differentiation and apoptosis will be held in May 1992. The main topics include: gene expression and differentiation, growth factors, oncogene products, models of differentiation (hematological, skin, immunological, neural) neuropeptides, signal transduction, retinoic acid receptors, active oxygen pathway, and programmed cell death (apoptosis). *Speakers already confirmed:* P. Calissano (I), L. Chieco-Bianchi (I), G. Ciliberto (I), F. Clementi (I), P. Comoglio (I), C. M. Croce (USA), J. Di Giovanna (USA), C. D'Onofrio (I), S. Ferrari (I), L. Fesus (H), A. Finazzi-Agro' (I), R. Horvitz (USA), M. Israel (USA), J. T. Keamshead (UK), R. A. Knight (UK), S. L. Lightman (UK), S. Parodi (I), M. Piacentini (I), U. Reichart (F), R. Revoltella (I), M. Santoro (I), M. Schwab, (FRG), S. Severin (USSR), R. Sitia (I), P. Steinert (USA), C. Thiele (USA), and A. Wyllie (UK). *General information:* Persons wishing to attend the course should write to: Scientific Secretary, Prof. Luisa Massimo and Dr. Gian Paolo Tonini, Department of Pediatric Hematology and Oncology, G. Gaslini Children's Hospital, L. go G. Gaslini 5, I-16148 Genova Quarto, Italy.