

Flow Cytometric Analysis of Phagocytosis, Respiratory Burst, Intracellular pH, and Cytosolic Free Calcium of Granulocytes of Post-traumatic and Septic Patients *

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Sepsis and trauma-related organ failure is intimately related to granulocyte functions. Granulocytes protect the organism against bacterial invasion but may also cause endothelial damage due to the release of reactive oxygen intermediates and proteolytic enzymes. The purpose of this study was the analysis of cell-biochemical parameters of granulocytes by flow cytometry and the use of the multifunctional parameter pattern for the prediction of sepsis- and trauma-related pulmonary and cardiovascular organ failure.

Materials and Methods

Buffy coat leukocytes were prepared from 236 samples of heparinized (10 U/ml) venous blood obtained serially from 55 patients of an intensive care unit. The leukocytes were sedimented at the erythrocyte/plasma interface by centrifugation at 200 g and 4 °C for 10 min. The surface layer of cells was carefully removed and resuspended in the supernatant plasma to a final concentration of 3.5×10^7 leukocytes/ml.

A suspension of 7×10^9 *E. coli* K12 bacteria (Sigma, Deisenhofen, FRG) in HEPES-buffered saline (HBS) (0.15 M NaCl, 5 mM HEPES, Serva, Heidelberg, FRG, pH 7.35) was prepared as described earlier [1]. One hundred microliters of the leukocytes suspended in autologous plasma were incubated with 10 µl of the *E. coli* suspension at 37 °C. Controls were incubated with HBS alone. Aliquots of 30 µl were taken at 15, 30, and 60 min respectively, diluted with 3 ml cold HBS, and stored on ice.

The phagocytic samples and control samples were each divided into five samples and incubated with different stains. Stock solutions of all dyes were made in *N,N*-dimethylformamide (DMF) (Merck, Darmstadt, FRG). Phagocytosis and degradation of bacterial DNA were measured as described in [1] after staining of the samples for 15 min at 4 °C in the presence of 8 µg/ml acridine orange (AO) (stock solution 0.4 mg/ml, Sigma). Intracellular pH and esterase activity were measured following 30 min incubation of the samples at 4 °C in the presence of 82 µM 1,4-diacetoxy-2,3-dicyanobenzene (ADB) (stock solution 4.1 mM, Paesel, Frankfurt, FRG), which is cleaved intracellularly by esterases to the pH indicator 2,3-dicyano-hydroquinone (DCH) and acetate [2]. Cytosolic-free calcium was measured after 30 min incubation at 37 °C with INDO-1 AM (stock so-

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lution 1 mM, Calbiochem, Frankfurt, FRG) at a final concentration of 2 μ M [3]. The phagosomal peroxidase-mediated hydrogen peroxide metabolism was measured after staining for 15 min at 37 °C in the presence of 10 μ M 2',7'-dichlorofluorescein diacetate (stock solution 10 mM, Serva, Heidelberg, FRG), which following cleavage is oxidized intracellularly to 2',7'-dichlorofluorescein (DCF) [4]. NADPH oxidase activity was measured through the intracellular oxidation of hydroethidine (HE) (stock solution 63.5 mM, Polyscience, St. Goar, FRG) to ethidium bromide (EB) after incubation for 15 min at 37 °C at a concentration of 160 μ M HE [5].

For the analysis of maximal achievable respiratory burst activity 10 μ l of the leukocyte suspension was diluted with 1 ml HBS, prestained for 15 min at 37 °C in the presence of 10 μ M DCFH-DA and/or 160 μ M hydroethidine, and stimulated for an additional 30 min at 37 °C with 150 nM phorbol 12-myristate 13-acetate (PMA) (stock solution 1.5 mM in DMF, Sigma).

Propidium iodide (PI) (Sigma) was added at a final concentration of 60 μ M 3 min prior to the flow-cytometric measurement to counterstain the DNA of dead cells.

The electrical cell volume and two fluorescences of each cell were measured simultaneously with a Fluvo-Metricell flow cytometer (HEKA-Elektronik, Lambrecht/Pfalz, FRG). The electrical cell volume was determined in a hydrodynamically focused cylindrical sizing orifice 80 μ m in diameter and 80 μ m in length at an electrical current of 0.15 mA using HBS as the sheath fluid. Fluorescence was excited with a HBO-100 high-pressure mercury arc lamp (Osram, Augsburg, FRG). DCH and INDO-1 fluorescence were excited between 340 and 400 nm and emission collected between 420 and 450 nm and above 500 nm. The fluorescences of AO, DCF, and EB were excited between 450 and 500 nm and measured between 500 and 530 nm and above 590 nm. The list-mode data were evaluated by the DIAGNOS1 program system for calculation, display, and databasing of flow cytometric data [6]. Between 12 and 24 parameters were automatically calculated for each measurement and transferred to a self-learning data base.

The prospectively identified patients were classified for each day of the study on the basis of pulmonary and/or cardiovascular organ failure of infectious or traumatic origin into four categories: (T) post-traumatic organ failure within 6 days following a trauma with a mean trauma score > 21 [7], (S) septic organ failure according to the definition of Montgomery et al. [8] (in addition either an infectious focus or a positive blood culture was present), (M) transition state, or (N) stable pulmonary and cardiovascular function after recovery from organ failure. The significance of the differences between groups was evaluated using the non-parametric Kruskal-Wallis one-way analysis of variance. A discriminant analysis based on the stepwise reduction of Wilks' lambda was calculated using the SPSS/PC+ package (SPSS, Chicago, USA).

Results and Discussion

Twenty-three of the 55 patients of this study developed at least one septic episode and 15 of the patients had post-traumatic organ failure in the absence of bacterial

Table 1. Functional parameters of phagocytosing and phorbol-ester-stimulated granulocytes as determined by flow cytometry

Parameters	Min	Stimulant	Clinical classification of the patients			Significance
			Normal (n = 146)	Sepsis (n = 28)	Trauma (n = 22)	
Cell volume (% of control)	30	<i>E. coli</i>	139.2 ± 1.8 ^b	140.5 ± 3.5	158.9 ± 7.8	0.037**
	90	<i>E. coli</i>	126.4 ± 1.6	129.0 ± 4.0	140.3 ± 7.3	0.313
AO green fluorescence (% of control)	30	<i>E. coli</i>	123.4 ± 2.3	122.2 ± 5.0	125.4 ± 4.9	0.783
	90	<i>E. coli</i>	134.5 ± 2.0	130.4 ± 5.1	135.1 ± 6.0	0.754
Internal pH	30	-	7.53 ± 0.02	7.64 ± 0.05	7.46 ± 0.06	0.059
	30	<i>E. coli</i>	7.47 ± 0.03	7.60 ± 0.05	7.35 ± 0.07	0.017**
Dead cells (% of leukocytes)	30	-	11.9 ± 0.7	11.3 ± 1.4	17.8 ± 3.8	0.040**
	30	<i>E. coli</i>	12.8 ± 0.9	11.5 ± 1.3	16.6 ± 4.2	0.443
Granulocytes (% of leukocytes)	0	-	66.2 ± 1.1	73.6 ± 1.9	76.8 ± 2.4	0.000**
			Normal (n = 27)	Sepsis (n = 5)	Trauma (n = 2)	
Internal Ca ² (F1/F2 ratio)	30	-	14.1 ± 0.2 ^b	14.9 ± 0.6	15.5 ± 0.1	0.110
	30	<i>E. coli</i>	14.5 ± 0.2	15.5 ± 0.6	16.2 ± 0.6	0.049**
DCF fluorescence (% of control)	15	<i>E. coli</i>	401.0 ± 36.6	280.3 ± 50.7	153.4 ± 19.1	0.049**
	30	PMA	698.4 ± 66.4	723.0 ± 112.0	249.8 ± 9.5	0.061
EB fluorescence (% of control)	15	<i>E. coli</i>	174.0 ± 9.6	150.4 ± 9.9	176.8 ± 9.1	0.442
	30	PMA	333.2 ± 10.4	361.3 ± 16.3	371.6 ± 21.6	0.222

** $P < 0.05$; Kruskal-Wallis one-way analysis of variance.

^a Classification as described in "Methods." Groups (M) and (N) are combined.

^b ± SEM.

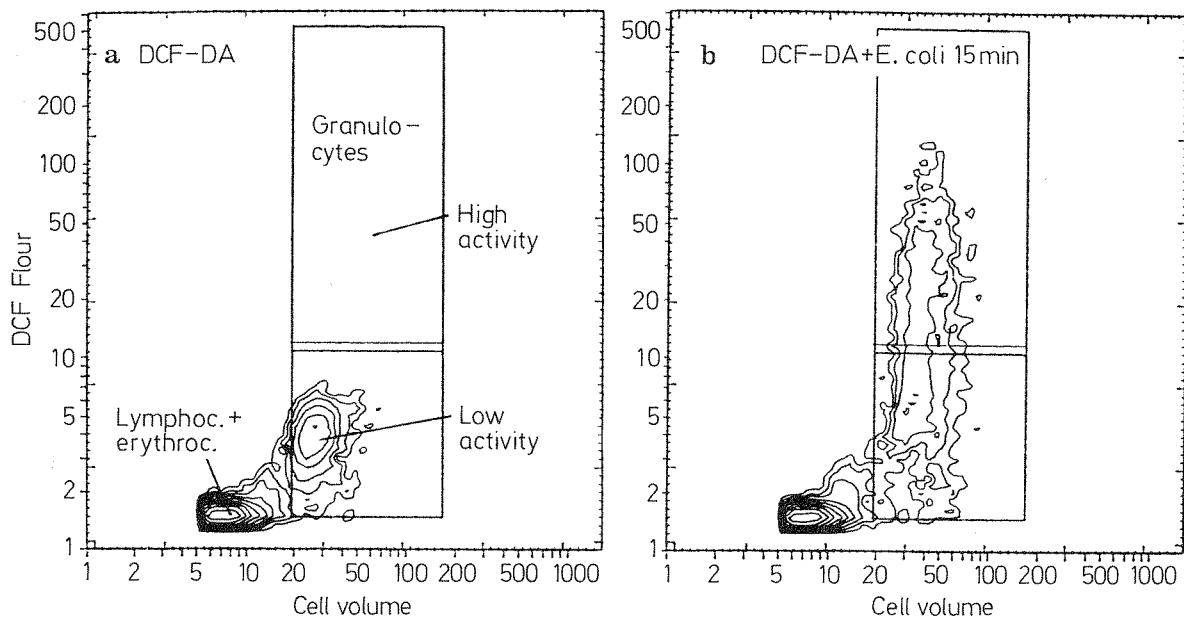


Fig. 1 a, b. Cell volume versus 2',7'-dichlorofluorescein-fluorescence of buffy coat leukocytes after 15 min incubation at 37 °C in the absence **a** or presence **b** of *E. coli*. The phagosomal peroxide level is increased after phagocytosis of bacteria. Dead cells are excluded from the display through gating on the simultaneously measured red fluorescence of DNA stained with PI

infection. Four patients developed sepsis following post-traumatic organ failure. Thirty-three of the 236 samples were obtained during septic episodes and 24 samples were obtained during post-traumatic organ failure.

Twenty-two of the 49 parameters of granulocyte function obtained by processing the flow-cytometric list mode with the DIAGNOS1 program system showed significant differences between samples obtained from septic patients, patients with post-traumatic organ failure, and patients in stable condition after recovery from organ failure. Granulocytes from post-traumatic patients were discriminated by increased phagocytosis of *E. coli* as measured by increased cell volume during incubation with *E. coli* and increased green fluorescence due to the ingested bacterial DNA (Table 1). The intracellular pH of the granulocytes was more acidic and more cells died during phagocytosis. The cytosolic-free calcium concentration was increased and peroxidase activity was decreased during phagocytosis (Fig. 1) and after stimulation with PMA (Fig. 2) despite unchanged NADPH oxidase activity. Granulocytes from septic patients had an alkaline pH but the mean uptake of bacteria was unchanged. NADPH oxidase activity and peroxidase activity were both depressed in phagocytosing but not in PMA-stimulated cells, which may reflect a defect in the activation cascade of the respiratory burst during sepsis.

Three canonical discriminant functions derived from stepwise variable selection based on the reduction of Wilks' lambda were used for the classification of granulocytes according to this functional pattern. Ninety percent of the post-traumatic samples and 78% of the septic samples were correctly classified by this procedure, the remainder being misclassified as transition state. No samples from patients in the (N) state were misclassified as septic or post-traumatic. This dis-

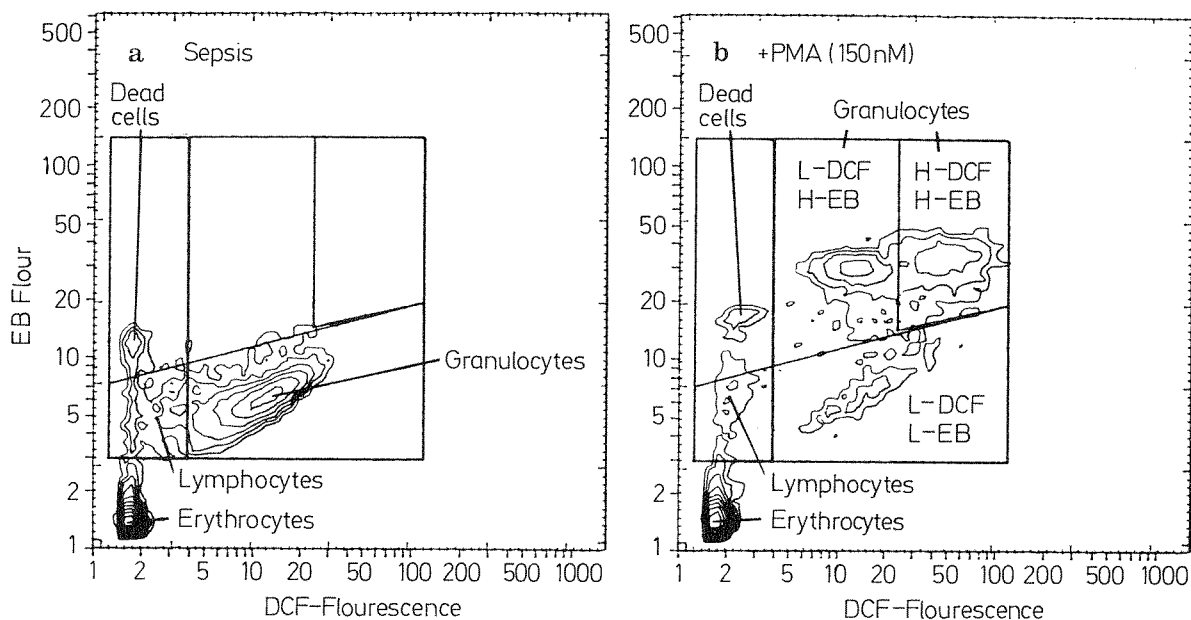


Fig. 2 a, b. 2',7'-Dichlorfluorescein fluorescence versus ethidium bromide fluorescence of buffy coat leukocytes of a septic patient incubated with 2',7'-dichlorfluorescein diacetate and hydroethidine without **a** or with **b** 150 nM PMA. More than 95% of the leukocytes are granulocytes. The simultaneous measurement of phagosomal H_2O_2 and NADPH oxidase activity reveals three subpopulations of granulocytes with different activity upon PMA stimulation

Table 2. Automated 3-day advance classification of intensive care patients using flow cytometric parameters of granulocyte function

Clinical diagnosis 3 days later	<i>n</i>	diagnosis/SPSS classification			
		Post- traumatic organ failure	Septic organ failure	Transition state	Normal
Post-traumatic organ failure	8	100.0%	0.0%	0.0%	0.0%
Septic organ failure	12	0.0%	91.7%	8.3%	0.0%
Transition state	94	1.1%	7.4%	87.2%	4.3%
Normal	23	0.0%	4.3%	0.0%	95.7%

Classification by three canonical discriminant functions based on stepwise minimization of Wilks' lambda. Clinical diagnosis as described in "Methods."

crimination was even improved when the discriminant analysis was run against the clinical classification on the 3rd day after the analysis of granulocyte function. One hundred percent of the episode could be predicted 3 days in advance on the basis of flow-cytometric parameters (Table 2).

The multifunctional analysis of cell-biochemical parameters by flow cytometry revealed significant early alterations of granulocyte function in septic and post-traumatic patients. The pattern of granulocyte function had a high predictive value for the progress of the disease and should be of value for the analysis of early effects of medical intervention on granulocyte function.

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