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Dihydrorhodamine 123: a New Flow Cytometric Indicator for Respiratory Burst Activity in Neutrophil Granulocytes

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Granulocytes phagocytose microorganisms and degrade them inside the phagosome by the action of reactive oxygen metabolites, proteases and antibacterial proteins [1, 2]. The production of superoxide anion by a membrane-bound NADPH-oxidase is central for the intracellular oxidative attack of the ingested material. Failure of superoxide anion production during phagocytosis results in severe infection [3]. Extracellular release of superoxide anion and its dismutation product hydrogen peroxide by granulocytes following activation by immune complexes or complement may cause endothelial damage, e.g., in the adult respiratory distress syndrome [4] or in rheumatic diseases [5].

2′,7′-Dichlorofluorescin diacetate (DCFH-DA) has been used for the measurement of intracellular reactive oxygen metabolite production in single granulocytes by flow cytometry [6]. Nonfluorescent 2′,7′-dichlorofluorescin (DCFH) is cleaved from the membrane-permeable DCFH-DA by intracellular esterases and accumulates in vital cells. Stimulation of the respiratory burst by the tumor promotor phorbol 12-myristate 13-acetate (PMA) or phagocytosis of bacteria results in high cellular fluorescence intensities due to intracellular oxidation of DCFH to fluorescent 2′,7′-dichlorofluorescein (DCF). Less potent stimuli, such as the chemotactic peptide for-met-leu-phe (FMLP), however, generate only barely measurable DCF signals. Minor burst stimulation is, however, of great interest for the determination of the effect of physiological stimuli on granulocytes.

It was the goal of the present study to develop a more sensitive method for the flow-cytometric measurement of respiratory burst activity. Dihydrorhodamine 123 (DHR), an uncharged and nonfluorescent derivative of the laser dye rhodamine 123 (R123) was found to be a nontoxic and about threefold more sensitive indicator of granulocyte respiratory burst activity than DCFH-DA. DHR is oxidized intracellulary during the respiratory burst to brightly fluorescent R123.

DHR was synthesized according to [7] and dissolved in N,N-dimethylformamide (DMF) (Merck, Darmstadt, FRG) at 15 mg ml^{-1} (43.3 m*M*). Leukocytes were obtained as supernatant following sedimentation of 3 ml of heparinized (10 U ml^{-1}) venous blood for 40 min at 1 *g* on 3 ml of Ficoll (density $d = 1.077$, Eurobio, Paris, F). Fifty μl of supernatant plasma containing the leukocytes were incubated for 15 min at 37 °C with 5 μl of a suspension of *E. coli* K12 (Sigma, Deisenhofen, FRG). *E. coli* were obtained from stationary cultures in RPMI 1640, washed twice with 50 ml HEPES-buffered saline (0.15 *M* NaCl, 5 m*M* HEPES, Serva, Heidelberg, FRG, pH 7.35) (HBS) and resuspended in HBS at 7×10^9 bacteria per ml. Ten-μl aliquots of the phagocytosis assay were diluted with 1 ml HBS and incubated for a further 15 min at 37 °C with either 1 μl of a 43.3-m*M* DHR-stock solution or 20 μl of a 500-μM working solution of DCFH-DA, obtained by diluting a 10-m*M* stock solution in DMF with HBS. Twenty μl of a 3-m*M* propidium iodide (PI) (Sigma) solution in HBS were added 3 min prior to the flow-cytometric measurement to counterstain the DNA of dead cells.

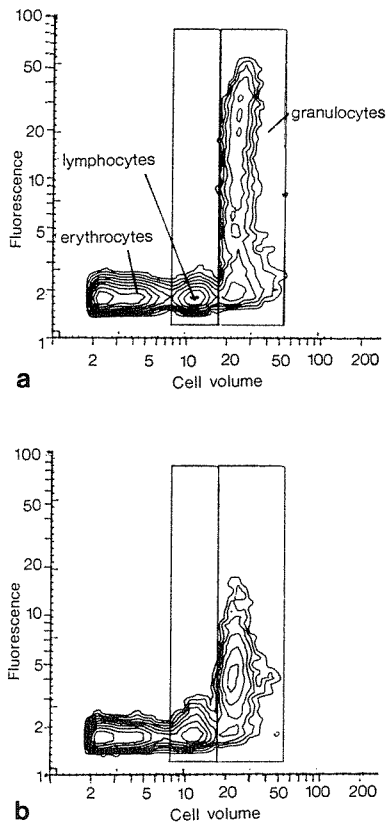

Fig. 1. Cell volume versus R123-fluorescence (a) or DCF-fluorescence (b) of leukocytes stimulated with $10^{-6}M$ N-formyl-met-leu-phe in the presence of 5 μg ml^{-1} cytochalasin B. DHR-stained granulocytes show significantly higher fluorescence (mode = 20 relative fluorescence units) than DCFH-DA-stained cells (four relative fluorescence units). The graphs are standardized to the maximum logarithmic channel contents (100 %). Contour lines were drawn in linear steps of 10 % downwards. The histograms contain 30039 (a) resp. 23690 (b) cells with 2312 and 1633 cells in the maximum channel

The electrical cell volume, the R123 green fluorescence (500 – 530 nm) and PI red fluorescence (590 – 700 nm) of each cell were simultaneously measured in a Fluvo-Metricell flow cytometer (HEKA-Elektronik, Lambrecht/Pfalz, FRG) [8]. The electrical cell volume was determined in a hydrodynamically focused cylindrical sizing orifice of 80 μm diameter and 80 μm length at an electrical current of 0.15 mA using HBS buffer as sheath fluid. Fluorescence was excited between 450 and 500 nm with an HBO-100 high-pressure mercury arc lamp (Osram, Augsburg, FRG). The list mode data were evaluated by the DIAGNOS1 program system for calculation, display and data basing of flow-cytometric data [9].

Granulocytes were strongly stimulated by the phagocytosis of bacteria and showed bright R123 green fluorescence. Their fluorescence was 20-fold higher than that of controls incubated in the absence of bacteria. Only an eightfold increase of fluorescence was observed with DCFH-DA. Lymphocytes, which do not exhibit respiratory burst activity, did not increase their green fluorescence upon stimulation. This showed both the specificity of DHR and the good cellular retention of R123, which was probably due to the intracellular accumulation of R123 at mitochondrial binding sites [10].

The effect of the weakly stimulating chemotactic peptide FMLP on DHR oxidation was studied in a second experiment. Ten μl of the suspension of leukocytes in plasma were diluted with 1 ml HBS supplemented with 1.3 m*M* Ca^{2+}. One μl of a 5-mg ml^{-1} solution of cytochalasin B (Sigma) in DMF was added to enhance the FMLP-induced responses in neutrophils [11]. The cells were stained for 15 min as described above and were incubated for a further 15 min at 37 °C following addition of 10 μl of 10^{-4} *M* FMLP (Sigma) in HBS, which had been prediluted from a 10-m*M* stock solution in dimethyl sulfoxide (Merck). Five μl of a 3-m*M* PI solution were added 3 min prior to the flow-cytometric measurement.

Granulocytes showed an 11-fold increase of R123 green fluorescence (Fig. 1a), when compared to a control without FMLP. This was higher than the threefold increase of DCF fluorescence in a DCFH-DA-stained sample (Fig. 1b). Furthermore, the wide heterogeneity of the FMLP-induced respiratory burst of granulocytes was only visible with the DHR stain.

The DHR method is significantly more sensitive for the flow-cytometric assay of respiratory activity in granulocytes than the DCFH-DA procedure. The described method is of value for the sensitive analysis of granulocyte function with physiological stimuli such as FMLP, activated complement, colony-stimulating factors, platelet-derived growth factor or interferons.

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