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FLOW CYTOMETRIC CHARACTERIZATION OF OXIDATIVE PROCESSES IN NEUTROPHILS AND MONOCYTES WITH DIHYDRORHODAMINE 123, 2',7'-DICHLOROFLUORESCIN AND HYDROETHIDINE

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INTRODUCTION

Neutrophils and monocytes release reactive oxygen metabolites such as superoxide anion, hydrogen peroxide and hypochlorous acid upon stimulation. High intracellular and extracellular levels of oxidants are required for the microbicidal and tumoricidal action of the phagocytes. Activation of the respiratory burst may lead to endothelial damage in non-infectious diseases like the adult respiratory distress syndrome, rheumatoid arthritis or myocardial reperfusion injury. The aim of this study was to characterize the intracellular processes in resting and stimulated neutrophils and monocytes using three fluorogenic indicators: dihydrorhodamine 123 (DHR),[1] 2',7'-dichlorofluorescin (DCFH)[2] and hydroethidine (HE).[3]

METHODS AND RESULTS

Cell-free experiments. The oxidation of DHR (Molecular Probes, Eugene, OR, USA) to rhodamine 123 (R123), DCFH (the intracellular cleavage product of 2',7'-dichlorofluorescin diacetate, Serva Feinbiochemica, Heidelberg, FRG) to 2',7'-dichlorofluorescein (DCF), and HE (Polysciences, St. Goar, FRG) to ethidium bromide (EB) was characterized in a cell-free assay by incubation with defined oxidants. DHR and DCFH were neither oxidized by a large excess of superoxide anion nor by hydrogen peroxide in contrast to HE which was significantly oxidized in the presence of superoxide anion (Fig. 1). All three substrates were oxidized by hydrogen peroxide in the presence of horseradish peroxidase (HRP).

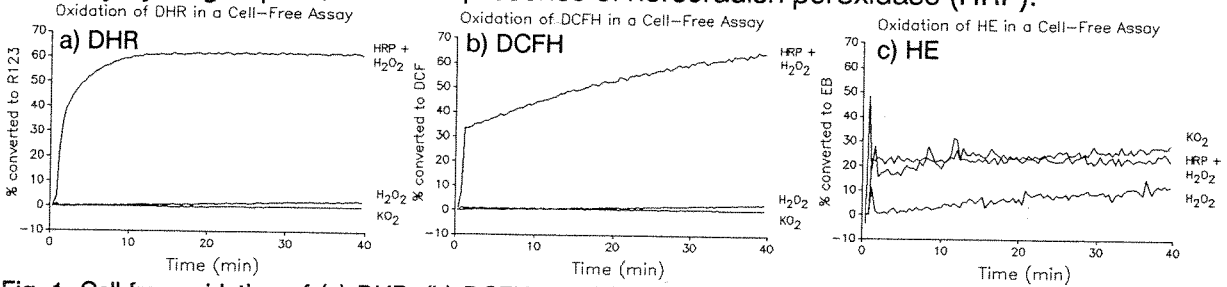

Fig. 1. Cell-free oxidation of (a) DHR, (b) DCFH and (c) HE in the presence of KO_2, a source of O_2^-, or H_2O_2 with or without horseradish peroxidase.

Cellular experiments. The intracellular oxidation of 40 μM DHR,[1] 10 μM DCFH[2,3] and 160 μM HE[3] by resting leukocytes and leukocytes stimulated with viable *E. coli*, 150 nM phorbol 12-myristate 13-acetate (PMA), 10^{-6} M *N*-formyl-Met-Leu-Phe (FMLP) or 100 μg/ml concanavalin A (ConA) was measured simultaneously with electrical cell volume in a FLUVO-II flow cytometer (HEKA-Elektronik, excitation: 400 - 500 nm, R123 or DCF emission: 500 - 530 nm, EB emission: 590 - 700 nm).

DHR stained samples when compared to DCFH showed a higher green fluorescence of stimulated neutrophils and monocytes but a lower fluorescence of unstimulated cells and lymphocytes indicating high sensitivity and specificity for respiratory burst activity (Fig. 2). The intracellular oxidation of HE to red fluorescent EB was a less sensitive indicator of respiratory burst activity. Higher spontaneous oxidation of HE by unstimulated monocytes and lymphocytes than neutrophils indicated additional oxidation not related to the respiratory burst. The decreased oxidation of DCFH but unchanged oxidation of HE in PMA-stimulated

neutrophils simultaneously incubated with DCFH-DA and HE suggested oxidation of HE at an earlier step in the cascade of intracellular oxidant formation during the respiratory burst.

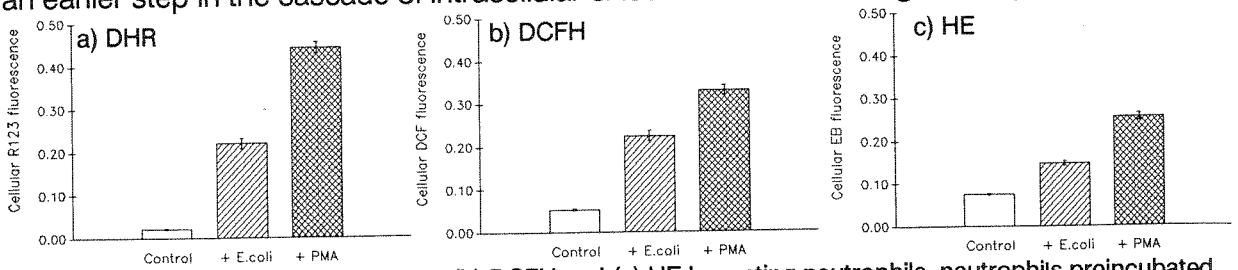

Fig. 2. Intracellular oxidation of (a) DHR, (b) DCFH and (c) HE by resting neutrophils, neutrophils preincubated with *E. coli* for 10 min or PMA-stimulated (30 min) neutrophils. Mean ± SEM (n = 58).

Monocytes had a lower respiratory burst activity than neutrophils with a higher response to *E. coli* (Fig. 3) than to PMA, high responses to ConA but a nearly undetectable response to FMLP. The respiratory burst of monocytes in contrast to neutrophils was inhibited rather than primed by prestimulation with 2 μM ionomycin (5 min) indicating differences of intracellular signal transduction between both cell types.

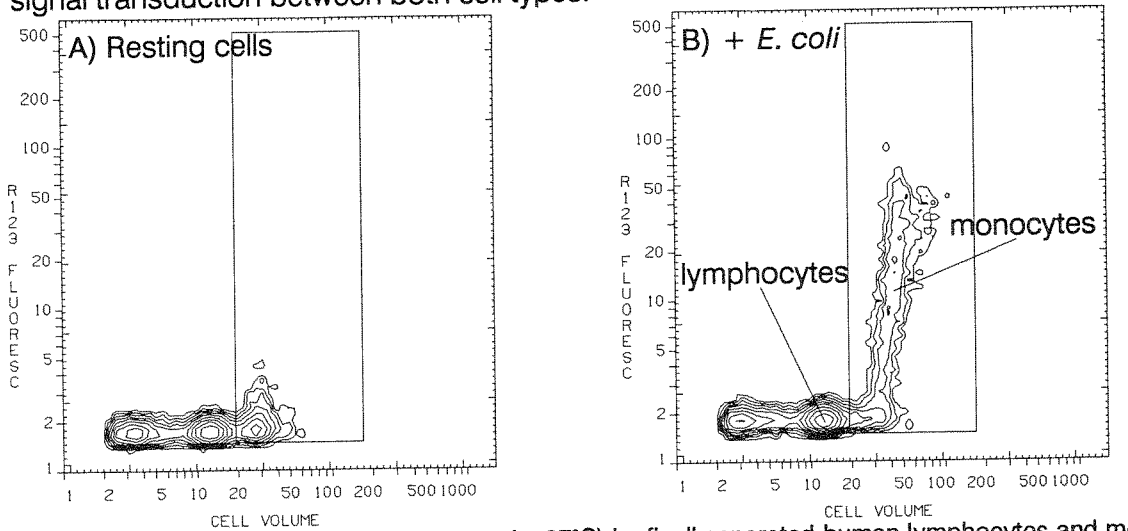

Fig. 3. Intracellular oxidation of DHR (40 μM, 15 min, 37°C) by ficoll-separated human lymphocytes and monocytes without (A) or with (B) *E. coli* preincubation for 10 min.

CONCLUSIONS

The respiratory burst activity of neutrophils and monocytes can be measured through the intracellular oxidation of DHR to R123, DCFH to DCF or HE to EB. The hydrogen peroxide-peroxidase sensitive DHR is the most sensitive substrate for the determination of respiratory burst activity of neutrophils and monocytes. The low fluorescence of lymphocytes indicates high specificity of DHR oxidation for the microbicidal oxidative system of phagocytic cells. The superoxide anion sensitive oxidation of HE to EB in resting and stimulated leukocytes can be used as an additional parameter of cellular activation. Simultaneous measurement of the intracellular oxidation of DCFH to green fluorescent DCF and of HE to red fluorescent EB resolves functionally heterogeneous subpopulations of neutrophils in infected patients.[4]

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