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Letter to the editors

Flow cytometric measurement of the respiratory burst activity of phagocytes using dihydrorhodamine 123

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Dear Editors,

A recent paper by Emmendörffer et al. (1990) published in the Journal of Immunological Methods described the adaptation to a FACScan flow cytometer (Becton Dickinson, San José, CA) in an assay (Rothe et al., 1988; Rothe and Valet, 1990c) for the determination of the respiratory burst activity of neutrophils using dihydrorhodamine 123 (DHR).

Oxidation of nonfluorescent DHR to the green fluorescent rhodamine 123 (R123) was highly specific for respiratory burst activity as it occurred in neutrophil subpopulations only of heterozygous carriers of chronic granulomatous disease (e.g., Fig. 4 in Emmendörffer et al., 1990). The relatively low yield of R123 green fluorescence in neutrophils stimulated with phorbol 12-myristate 13-acetate (PMA) and the complete inhibition of respiratory burst activity upon the addition of DHR prior to stimulation with PMA are contradictory to results obtained with the original method. This inhibition of the fluorescence response was caused by the high concentration of *N*,*N*-dimethylformamide (DMF) (1%) used in the modified assay.

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Optimal performance of the DHR assay using a 488 nm argon laser-equipped flow cytometer is obtained by preincubating 1×10^6 leukocytes/ml in 5 mM Hepes-buffered saline (0.15 M NaCl, pH 7.35) for 5 min with 1 μM DHR (stock solution 1 mM in DMF, predilution ten-fold in saline) followed by stimulation with 100 nM PMA (stock solution 1 mM in DMF) for 20 min. This typically results in a 170–210-fold increase of the green fluorescence (500–540 nm) of neutrophils with no detectable increase in lymphocyte fluorescence (Fig. 1*a*). Furthermore, the kinetics of the respiratory burst activity can be studied and neutrophil subpopulations with different time courses of hydrogen peroxide production can be observed (Fig. 2).

The high sensitivity of the DHR method becomes apparent when compared with the only 15–20-fold increase of 2′,7′-dichlorofluorescein green fluorescence of neutrophils incubated with 10 μM 2′,7′-dichlorofluorescein diacetate (DCFH-DA) (Fig. 1*b*) as proposed in an earlier published method (Bass et al., 1983). Furthermore, the DCFH-DA assay shows a significant non-specific increase of lymphocyte fluorescence, although lymphocytes do not express the NADPH oxidase of phagocytic cells. The specific analysis of the low and heterogeneous oxidative response of neutrophils to weak physiological stimuli such as the bacterial peptide *N*-formyl-Meth-Leu-Phe is, therefore, only possible with the DHR method

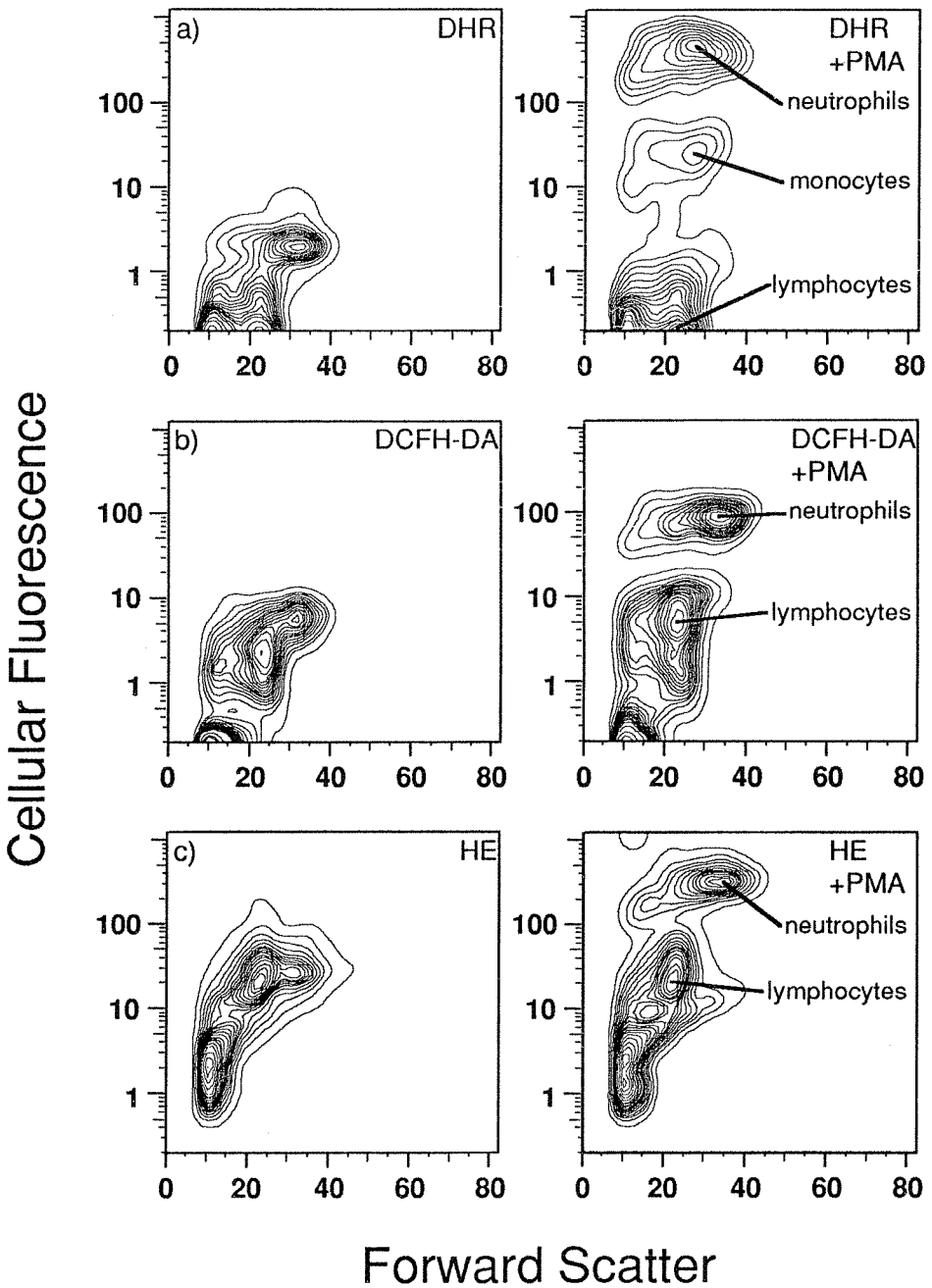

Fig. 1. Comparison of fluorogenic substrates for the respiratory burst activity of human neutrophils. Leukocytes were preincubated for 5 min at 37 °C either with 1 μM DHR (*a*), 10 μM DCFH-DA (*b*), or 10 μM HE (*c*) followed by addition of 100 nM PMA for 20 min. The increase of the green (*a*,*b*) and red (*c*) fluorescence of neutrophils is a measure of the respiratory burst activity. Increases of lymphocyte fluorescence in the DCFH-DA assay (*b*) indicate a non-specific reaction as they do not correspond to respiratory burst activity. The histograms were obtained from measurements on a FACStar^{PLUS} cell sorter using FACS/DESK software.

(Rothe and Valet, 1990b). This method proved to be more sensitive and reliable than other clinical, diagnostic and functional tests of the formation of oxidative products by neutrophils such as lucigenin- or luminol-dependent chemiluminescence, cytochrome *C* reduction or nitro blue tetrazolium reduction (Roesler et al., 1990a,b,c).

The 6–11-fold increase in the ethidium bromide red fluorescence (above 600 nm) of PMA-stimulated neutrophils preincubated with 10 μM hydro-

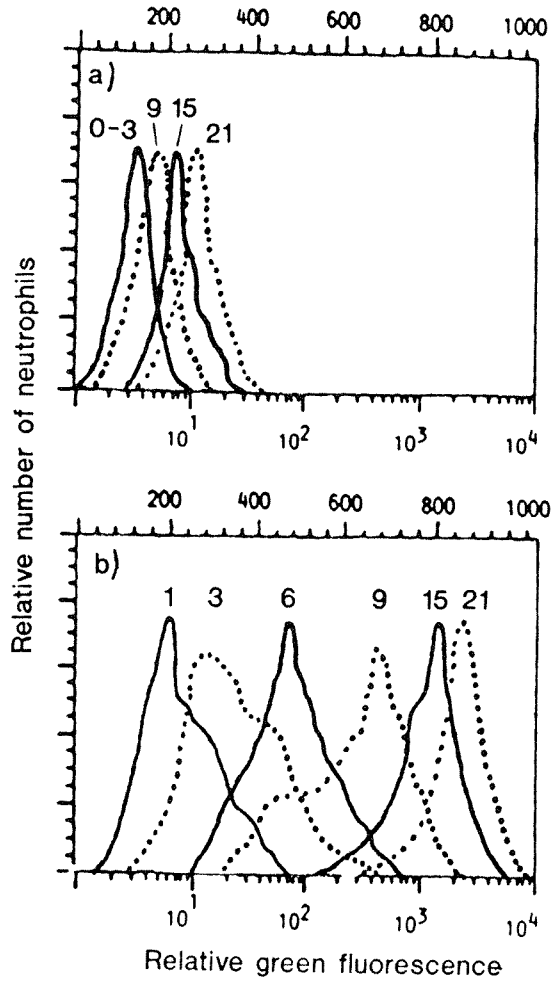

Fig. 2. Kinetic measurement of the respiratory burst activity of neutrophils incubated (*a*) with DHR alone or (*b*) with DHR and PMA. Numbers indicate minutes after addition of DHR. The histograms were obtained from measurements on a FACScan flow cytometer using FACScan research software.

ethidine (HE) can be used as a further indicator of respiratory burst activity (Rothe et al., 1990a). This method is especially interesting because HE is sensitive to oxidation by the superoxide anion (Rothe et al., 1990a) in contrast to the oxidation of 2′,7′-dichlorofluorescein (Bass et al., 1983) and DHR (Rothe and Valet, 1990c) by a hydrogen peroxide and peroxidase dependent mechanism.

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