[56] Flow Cytometric Assays of Oxidative Burst Activity in Phagocytes

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Introduction

Phagocytic cells such as neutrophils, monocytes, and macrophages are equipped with a specific enzymatic cascade for the production of reactive oxidants.[1] The release of superoxide anion, hydrogen peroxide,

[1] R. A. Clark, *J. Infect. Dis.* **161,** 1140 (1990).

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and hypochlorous acid into phagosomes and into the extracellular space during the oxidative burst is important for their microbicidal activity. Defects of reactive oxidant production, such as in the inherited chronic granulomatous diseases, are associated with severe bacterial infections. Phagocytic oxidants and the lysosomal proteases of phagocytic cells also play a role as mediators of tissue destruction in the inflammatory reaction.[2] The primary enzyme of the oxidative burst cascade, the NADPH oxidase, is a characteristic enzyme of phagocytic cells. On activation, the enzyme incorporates into the plasma or lysosomal membrane and catalyzes the transmembrane transport of electrons from NADPH to molecular oxygen. Superoxide anion, the product of the one-electron reduction of molecular oxygen, dismutates to hydrogen peroxide, which finally is converted by myeloperoxidase to long-lasting oxidants such as hypochlorous acid or chlorinated tauramines.

$$\mathrm{NADPH} + 2\,\mathrm{O_2} \xrightarrow{\text{NADPH oxidase}} \mathrm{NADP^+} + \mathrm{H^+} + 2\,\mathrm{O_2^-}$$

$$2\,\mathrm{O_2^-} + 2\,\mathrm{H^+} \xrightarrow{\text{superoxide dismutase}} \mathrm{H_2O_2} + \mathrm{O_2}$$

$$\mathrm{H_2O_2} + \mathrm{X^-} + \mathrm{H^+} \xrightarrow{\text{myeloperoxidase}} \mathrm{HOX} + \mathrm{H_2O}$$

$$\mathrm{X^-} = \mathrm{Cl^-}, \mathrm{Br^-}, \mathrm{I^-}, \mathrm{SCN^-})$$

Two other oxidants, namely, hydroxyl radical, originating from an iron-catalyzed reaction of O_2^- with H_2O_2,[3] a reaction of O_2^- with HOCl,[4] or a reaction of O_2^- with nitric oxide,[5] and singlet oxygen, generated from a water-induced dismutation of O_2^-,[6] or in a myeloperoxidase-dependent reaction,[7] may also play a role in the respiratory burst reaction. Whether a production of hydroxyl radical or singlet oxygen by neutrophils occurs under physiological reaction is unclear.[8–10]

Specific biochemical methods for the quantitation of the respiratory burst activity determine either the extracellular oxygen consumption or

[2] S. J. Weiss, *N. Engl. J. Med.* **320,** 365 (1989).
[3] J. V. Bannister and W. H. Bannister, *Environ. Health Perspect.* **64,** 37 (1985).
[4] C. L. Ramos, S. Pou, B. E. Britigan, M. S. Cohen, and G. M. Rosen, *J. Biol. Chem.* **267,** 8307 (1992).
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[6] E. J. Corey, M. M. Mehrotra, and A. U. Khan, *Biochem. Biophys. Res. Commun.* **145,** 842 (1987).
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[10] B. E. Britigan, J. S. Serody, M. B. Hayek, L. M. Charniga, and M. S. Cohen, *J. Immunol.* **147,** 4271 (1991).

the extracellular release of O_2^- and H_2O_2.[11] These assays require the isolation of phagocytic cells by density gradient centrifugation, which leads to an alteration of their functional properties.[12] Furthermore, the cellular heterogeneity cannot be analyzed by biochemical bulk assays. Flow cytometric methods, in contrast, permit the measurement of the oxidative burst activity in single cells. A widely used assay is based on the intracellular oxidation of nonfluorescent 2′,7′-dichlorofluorescin (DCFH) to green fluorescent 2′,7′-dichlorofluorescein (DCF) following enzymatic deacetylation of the membrane-permeable 2′,7′-dichlorofluorescin diacetate (DCFH-DA).[13] DCFH, in aqueous media, is oxidized by hydrogen peroxide in the presence of either peroxidase or ferrous iron, whereas no oxidation occurs in the presence of superoxide anion.[14] In cellular assays DCFH has the drawback of a low sensitivity in cells with a low esterase activity such as monocytes.[15] Furthermore, significant oxidation of DCFH has been reported in cells lacking NADPH oxidase such as keratinocytes[16] or platelets,[17] suggesting a low specificity of the reaction.

In an alternative method the intracellular oxidation of the membrane-permeable blue fluorescent substrate hydroethidine (HE) to red fluorescent ethidium bromide by a wide range of oxidants including superoxide anion and hydrogen peroxide in a peroxidase-dependent reaction has been described.[18] HE, similarly to DCFH, is not a very sensitive indicator of the oxidative burst activity as a high cellular fluorescence of neutrophils is induced only by potent stimuli, such as the protein kinase C ligand phorbol 12-myristate 13-acetate, or particulate stimuli, such as bacteria. The cellular response to low concentrations of physiological stimuli such as the bacterial peptide *N*-formyl-Met-Leu-Phe (FMLP) or cytokines such as tumor necrosis factor-α (TNF-α) is barely detectable with either DCFH or HE.

[11] J. A. Badwey, J. M. Robinson, M. J. Karnovsky, and M. L. Karnovsky, *in* "Handbook of Experimental Immunology, Volume 2: Cellular Immunology" (D. M. Weir, ed.), p. 501. Blackwell Scientific, Oxford, 1986.

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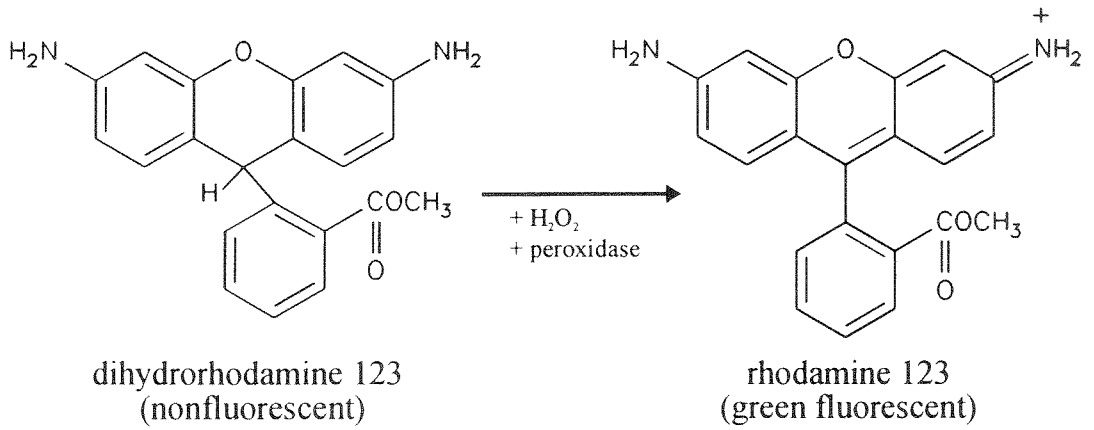

FIG. 1. Scheme of the oxidation of dihydrorhodamine 123.

A significantly higher sensitivity is obtained by the use of the nonfluorescent and membrane-permeable substrate dihydrorhodamine 123 (DHR)[19] (Figs. 1 and 2), which permits the detection of intracellularly generated H_2O_2 in phagocytic cells in a one-step reaction.[20,21] DHR is oxidized to the cationic rhodamine 123 which accumulates intracellularly owing to the electrically negative cytoplasmic and mitochondrial membrane potential. The method can be combined with the simultaneous counterstaining of dead cells with propidium iodide, staining of cellular surface antigens by immunofluorescence, and the analysis of other functional parameters such as intracellular calcium[22] or intracellular glutathione concentrations.[23]

Principle of Assay

Peripheral blood is depleted of erythrocytes by a 1 g sedimentation on top of Ficoll. The supernatant leukocyte-rich plasma is aliquoted, and leukocytes are loaded with the fluorogenic substrate dihydrorhodamine 123 for 5 min at 37°, followed by incubation with stimuli such as the cytokine TNF-α or the bacterial peptide FMLP for 5 to 20 min at 37°. The incubation is stopped on ice, dead cells are counterstained by propidium iodide, and the green fluorescence of rhodamine 123, which was

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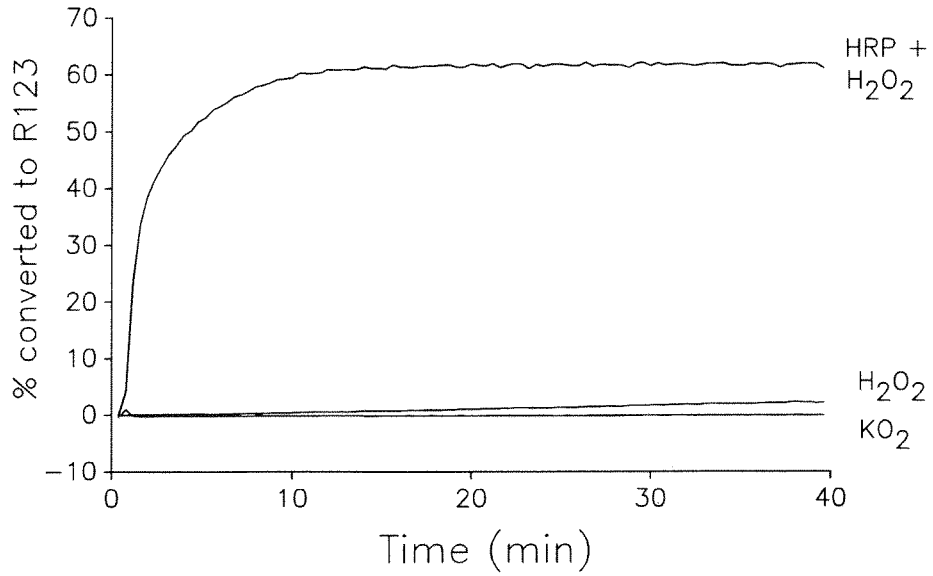

FIG. 2. Characterization of dihydrorhodamine 123 oxidation in a cell-free assay (HRP, horseradish peroxidase).

generated intracellularly in the presence of hydrogen peroxide, is analyzed by flow cytometry.

Materials: Stock and Working Solutions

Dulbecco's phosphate-buffered saline without sodium bicarbonate (Dulbecco's PBS, GIBCO, Grand Island, NY)

Dihydrorhodamine 123 (DHR, Molecular Probes, Eugene, OR, stock solution 1 m*M* in *N,N*-dimethylformamide, working solution 100 μM in Dulbecco's PBS); protect from light and store stock solution at −40°

Propidium iodide (PI, Serva, Heidelberg, Germany, stock solution 3 m*M* in phosphate-buffered saline)

Tumor necrosis factor-α, human, recombinant (TNF-α; Sigma, St. Louis, MO, 1 μg/ml in Dulbecco's PBS)

N-Formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP, Sigma, stock solution 1 m*M* in *N,N*-dimethylformamide, working solution 10 μM in Dulbecco's PBS)

Phorbol 12-myristate 13-acetate (PMA, Sigma, stock solution 1 m*M* in *N,N*-dimethylformamide, working solution 10 μM in Dulbecco's PBS)

Isolation of Leukocytes by Sedimentation of Erythrocytes on Ficoll. Leukocyte isolation procedures that depend on a lysis of erythrocytes or on the centrifugation of erythrocytes through density gradients lead to activation of leukocytes, resulting in a high spontaneous oxidative burst activity and increased responses to cellular stimulation. Therefore, blood

is depleted of erythrocytes by a method which avoids contact of leukocytes with any separation media. Undiluted heparinized (10 U/ml) whole blood (3 ml) is layered on top of 3 ml lymphocyte separation medium (Ficoll, density 1.077 g/ml). Erythrocytes will aggregate at the interface and sediment at room temperature without centrifugation.[24] After 40 min, the upper 800 μl of supernatant plasma is withdrawn, avoiding contact with the plasma fraction near the interface with the separation medium, and stored on ice. This will contain platelets and approximately 2×10^7/ml unseparated leukocytes in autologous plasma.

Assay Procedure

1. For chemotactic stimulation, put 1 ml Dulbecco's PBS, 20 μl leukocyte suspension, and 10 μl dihydrorhodamine working solution in a 12 × 75 mm polypropylene test tube (final dihydrorhodamine concentration 1 μM). Incubate for 5 min at 37°. Add 10 μl FMLP working solution (final FMLP concentration 100 nM) or PBS as a control incubation. Continue incubation, taking 250-μl aliquots at 5, 10, and 15 min after addition.

2. For the analysis of cytokine priming, put 1 ml Dulbecco's PBS, 20 μl leukocyte suspension, and 10 μl dihydrorhodamine working solution in a 12 × 75 mm polypropylene test tube (final dihydrorhodamine concentration 1 μM). Incubate for 5 min at 37°. Add 10 μl TNF-α solution (final TNF-α concentration 10 ng/ml) or PBS as a control incubation. After 5 min add 10 μl FMLP working solution (final FMLP concentration 100 mM). Continue incubation, taking 250-μl aliquots at 5, 10, and 15 min after addition.

3. For PMA stimulation, put 1 ml Dulbecco's PBS, 20 μl leukocyte suspension, and 10 μl dihydrorhodamine working solution in a 12 × 75 mm polypropylene test tube (final dihydrorhodamine concentration 1 μM). Incubate for 5 min at 37°. Add 10 μl PMA working solution (final PMA concentration 100 nM) or PBS as a control incubation. Continue incubation, taking 250-μl aliquots at 10, 20, and 30 min after addition.

4. For phagocytosis of bacteria, put 100 μl leukocyte suspension and 10 μl of suspension from a stationary culture of *Escherichia coli* K12 (5×10^9 bacteria/ml Dulbecco's PBS) in a 12 × 75 mm polypropylene test tube. Incubate at 37°. Take 20-μl aliquots at 5, 10, 15, and 20 min, dilute each with 1 ml ice-cold Dulbecco's PBS, and store on ice until staining. To stain, incubate 1 ml diluted cell suspension with 10 μl dihydrorhodamine working solution for 15 min at 37°.

[24] A. Böyum, *Scand J. Clin. Lab. Invest.* **21** (Suppl. 97), 1 (1968).

5. Counterstain dead cells by incubating 250 μl stained cell suspension with 5 μl of 3 mM propidium iodide for 3 min on ice (final PI concentration 60 μM).

6. Analyze rhodamine 123 green fluorescence (510 to 530 nm) and propidium iodide red fluorescence (above 600 nm) of 10,000 leukocytes for each sample on a flow cytometer using argon ion laser excitation at 488 nm or a high-pressure mercury arc lamp and a 470 to 500 nm bandpass filter.

Typical results of the assay are shown in Fig. 3. Neutrophils and monocytes show only a low spontaneous oxidative burst activity when

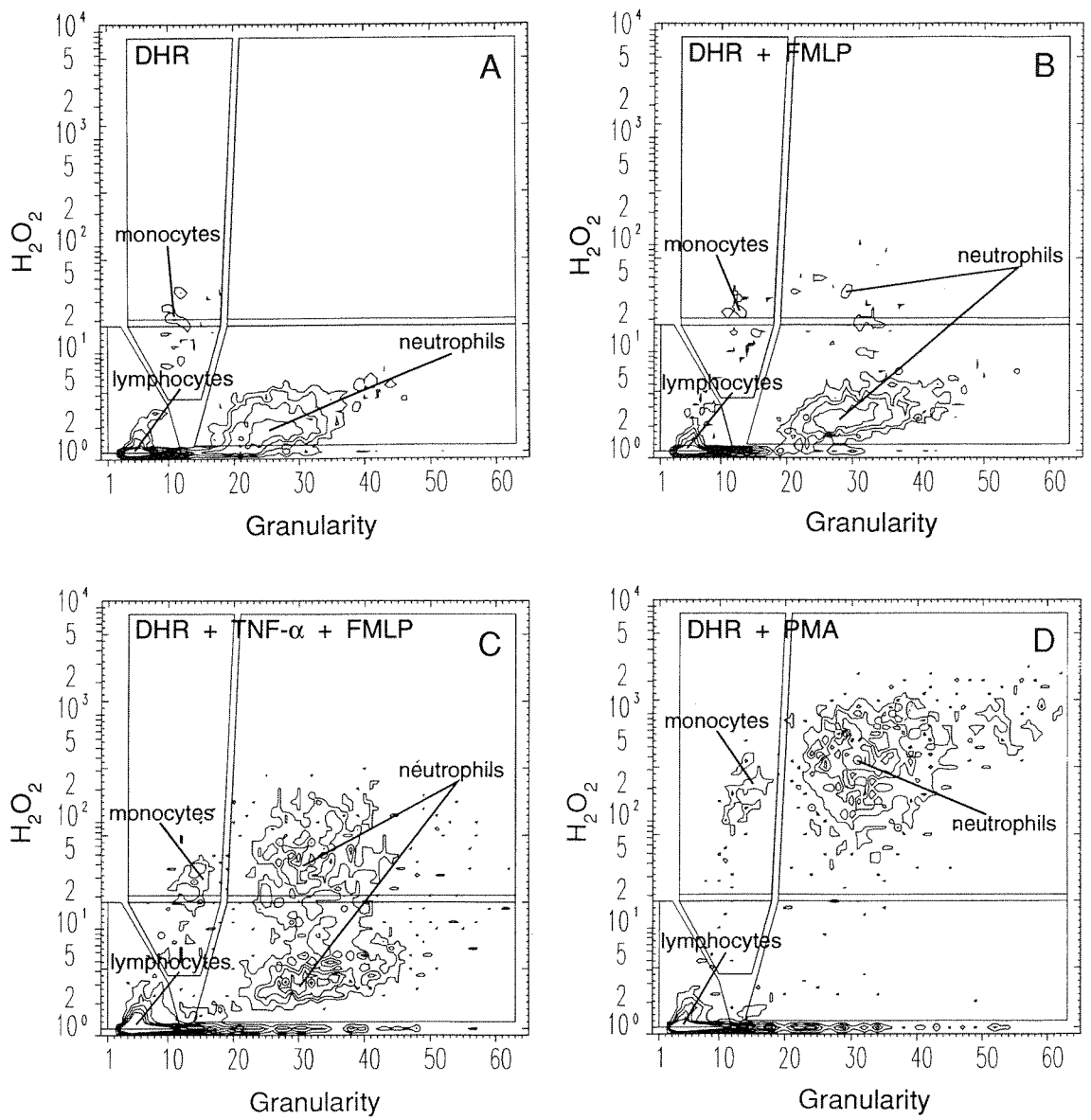

FIG. 3. Analysis of the oxidative burst response of neutrophils and monocytes in a whole blood assay using dihydrorhodamine 123.

artifactual stimulation by cellular isolation procedures or contact to polystyrene or glass surfaces are avoided (Fig. 3A). The chemotactic peptide FMLP (10^{-7} *M*) in blood samples from normal individuals induces only a low oxidative burst activity in neutrophils, which react in an all-or-none response (Fig. 3B). The subset of neutrophils showing a high oxidative burst activity is greatly enhanced in blood samples obtained during systemic bacterial infection or following *in vitro* priming of leukocytes with TNF-α (10 ng/ml) (Fig. 3C). The same response can be induced by increasing the concentration of FMLP (10^{-7} *M*). Stimuli that bypass the cytoplasmic signal transduction such as the tumor promoter PMA induce a homogeneous increase of oxidative burst activity in all neutrophils and monocytes (Fig. 3D) and may be used as a positive control or when the expression of the oxidative burst enzymes is analyzed such as in the diagnosis of chronic granulomatous disease.[25]

Modifications of Assay

Positive and Negative Controls

Extracellular hydrogen peroxide and a specific inhibitor of the NADPH oxidase may be used as positive and negative controls, respectively. As a positive control, DHR-loaded cells (1 μM DHR, 5 min, 37°) are incubated with 1 m*M* hydrogen peroxide for 15 min at 37°. This should result in a homogeneous high fluorescence of all cells containing peroxidase. To demonstrate specificity of the intracellular generation of rhodamine 123 fluorescence for the NADPH oxidase, phagocytic cells may be preincubated with the NADPH oxidase inhibitor diphenyl iodonium[26] (400 μM, stock 100 m*M* in dimethyl sulfoxide) for 20 min at 37° before stimulation. This should result in an over 90% inhibition of DHR oxidation.

Improved Discrimination of Leukocytes by Counterstaining

The fluorescence of unstimulated leukocytes loaded with DHR is near to autofluorescence, which only allows their identification based on cellular scatter characteristics or cell volume signals. Counterstaining of leukocytes with the esterase substrate carboxy-SNARF-1/AM or the DNA stain LDS-751 may be used to improve the discrimination of leukocytes, platelets, and erythrocytes, especially when the assay is performed in whole blood. Incubation of the cell samples with carboxy-SNARF-1/AM

[25] J. Roesler, M. Hecht, J. Freihorst, M. L. Lohmann-Matthes, and A. Emmendörffer, *Eur. J. Pediatr.* **150,** 161 (1991).

[26] A. R. Cross, *Biochem. Pharmacol.* **36,** 489 (1987).

(Molecular Probes, 0.1 μM for 25 min at 37°) simultaneously with DHR or the addition of the red fluorescent DNA stain LDS-751 (Exciton, Dayton, OH, 0.4 μM for 5 min at 37°) at the end of the incubation will result in a bright red fluorescence of all viable leukocytes, which can be used for proper gating on the various cell populations.

Improving Intracellular Retention of Rhodamine 123 by Fixation

Cellular staining is usually stable for at least 2 hr at 4° owing to the intracellular trapping of rhodamine 123 at mitochondrial binding sites. If prolonged stability of the assay samples is required, fixation with 1% (w/v) paraformaldehyde in PBS may be performed to maintain cellular staining.[27]

Combination with Immunofluorescence

The analysis of the oxidative burst response of leukocytes may be combined with the immunofluorescent staining of cellular surface antigens. To avoid interaction of the antibody staining with the functional analysis, leukocytes are first incubated with DHR and the appropriate stimuli as described above. Cells are washed with 3 ml PBS at 60 *g* for 5 min, resuspended in 50 μl PBS, and incubated with the fluorescent conjugated antibodies at 4° for 15 min. After washing twice with 3 ml PBS (60 *g*, 5 min) cells can be analyzed by flow cytometry. For 488-nm single laser excitation, preferentially long wavelength fluorophores such as *R*-phycoerythrin/Cy5 tandem conjugates or PerCP should be used for the conjugation of antibodies as no spectral overlap occurs to R123 fluorescence. Alternatively biotinylated antibodies may be combined with *R*-phycorerythrin/Cy5-streptavidin or PerCP-streptavidin. *R*-Phycoerythrin-conjugated antibodies may be added for dual-parameter immunofluorescence; however, the fluorescence compensation needs to be adjusted carefully.

Combination with Other Functional Parameters

The analysis of the oxidative burst response of phagocytic cells may be combined with the simultaneous analysis of other functional parameters such as the cytosolic free calcium level or the intracellular glutathione concentration. For the simultaneous analysis of intracellular calcium cells may be either counterstained with the red fluorescent calcium-sensitive substrate Fura red/AM using single laser excitation at 488 nm[28] or with

[27] R. P. Linke, V. Bock, G. Valet, and G. Rothe, *Biochem. Biophys. Res. Commun.* **176,** 1100 (1991).

[28] F. Lund-Johansen and J. Olweus, *Cytometry* **13,** 693 (1992).

indo-1/AM using dual laser excitation (365 nm/488 nm).[29] Simultaneous analysis of intracellular glutathione concentrations can be achieved using counterstaining with *o*-phthaldialdehyde.[30]

Alternative Methods

Alternative fluorogenic indicators for the oxidative burst activity such as DCFH and HE have a significantly lower sensitivity for the detection of oxidants when compared with DHR. The simultaneous analysis of the generation of green fluorescent DCF from the peroxidase-dependent oxidation of DCFH and of the red fluorescent ethidium bromide from the O_2^--sensitive substrate HE may be used for dual color analysis of different steps of the intracellular oxidative burst cascade.[16] Reduced oxidation of DCFH, but not of HE, was observed during sepsis, a disease known to be associated with the liberation of high amounts of myeloperoxidase from lysosomes through degranulation.

The DCF and HE labels also permit the convenient simultaneous analysis of the uptake of fluorescently labeled bacteria and oxidative burst production. Texas Red-labeled bacteria have been used in combination with DCFH in a method which depends on dual-laser excitation.[31] A more simple approach on a 488-nm single-laser flow cytometer consists of the combination of HE staining with fluorescein isothiocyanate (FITC)-labeled bacteria.[32]

Other flow cytometric methods for the analysis of the oxidative burst response are based on either the production of fluorescent formazans[33] or the quenching of cellular fluorescence by nitro blue tetrazolium.[34] Both methods, however, show comparatively low sensitivity, and the specificity of the assays has not been thoroughly characterized.

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