(f) Measurement of Phagosomal Hydrogen Peroxide Production with Dihydrorhodamine 123

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Outline

During the oxidative burst, phagocytic cells release superoxide anion through the membrane-bound NADPH oxidase. Hydrogen peroxide, produced by dismutation of superoxide anion, is the substrate for the myeloperoxidase-catalyzed oxidation inside the phagosome. The nonfluorescent dihydrorhodamine 123 is oxidized intracellularly in a peroxidase-dependent reaction to green fluorescent rhodamine 123. Stimulation of neutrophils by PMA or by phagocytosis of bacteria results in a 200- to 1200-fold increase of cellular green fluorescence. An increased oxidative burst response to the less potent bacterial peptide FMLP is a sensitive indicator for the preactivated state of neutrophils during sepsis or following exposure to cytokines.

Specimen: 3 ml heparinized human blood (10 U heparin/ml)

Reagents

HBSS without phenol red or bicarbonate, supplemented with 10 mM HEPES (pH 7.35)

dihydrorhodamine 123 (MW 346)
stock solution: 1 mM in DMF (346 µg/ml)
working solution: 100 µM (1:10 dilution of stock in HBSS)

propidium iodide (PI) (MW 668.4)
stock solution: 3 mM (2 mg/ml) in 5 mM HEPES-buffered saline (0.15 M NaCl, pH 7.35)

phorbol 12-myristate 13-acetate (PMA) (MW 616.8)
stock solution: 1 mM in DMF
working solution: 10 µM (1:100 dilution of stock in HBSS)

N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP) (MW 437.6)
stock solution: 1 mM in DMF
working solution: 10 µM (1:100 dilution of stock in HBSS)

Procedure

1. Layer 3 ml heparinized blood carefully on top of 3 ml lymphocyte separation medium. Allow erythrocytes to sediment for 40 minutes at room temperature.
2. Withdraw the upper 800 µl supernatant plasma and store on ice. This will contain platelets and approximately 2 x 10^7/ml unseparated leukocytes.
3. **For PMA stimulation**, put 1.00 ml HBSS, 20 µl cell suspension, and 10 µl dihydrorhodamine working solution in a 12 x 75 mm polypropylene test tube (final dihydrorhodamine concentration 1 µM). Incubate for 5 minutes at 37°C. Add 10 µl PMA working solution (final PMA concentration 100 nM). Continue incubation, taking 250 µl aliquots at 10, 20, and 30 minutes after addition.
4. **For chemotactic stimulation**, put 1.00 ml HBSS, 20 µl cell suspension, and 10 µl dihydrorhodamine working solution in a 12 x 75 mm polypropylene test tube (final dihydrorhodamine concentration 1 µM). Incubate for 5 minutes at 37°C. Add 10 µl FMLP working solution (final FMLP concentration 100 nM). Continue incubation, taking 250 µl aliquots at 5, 10, and 15 minutes after addition.
5. **For phagocytosis of bacteria**, put 100 µl cell suspension and 10 µl stationary culture *Escherichia coli* K12 suspension (5 x 10^9 bacteria/ml HBSS) in a 12 x 75 mm polypropylene test tube. Incubate

at 37°C. Take 20 µl aliquots at 5, 10, 15, and 20 minutes, dilute each with 1.00 ml cold HBSS, and store on ice until staining. To stain, incubate 1.00 ml diluted cell suspension with 10 µl dihydrorhodamine working solution for 15 minutes at 37°C.

6. Counterstain dead cells by incubating 250 µl stained cell suspension with 5 µl 3mM PI for 3 minutes on ice (final PI concentration 60 µM).
7. Run on flow cytometer.

Excitation: 488 nm (argon laser) or high pressure mercury arc lamp with 470-500 nm bandpass filter
Filters: 510-530 bandpass for rhodamine 123 green fluorescence
600 nm longpass for PI (dead cells)

Reagents

HBSS	Sigma Chemical Co.	# H-1387
Dihydrorhodamine 123	Molecular Probes	# D-632
Propidium iodide	Serva	# 33671
Phorbol 12-myristate 13-acetate	Sigma Chemical Co.	# P-8139
FMLP	Sigma Chemical Co.	# F-3506

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NOTES:

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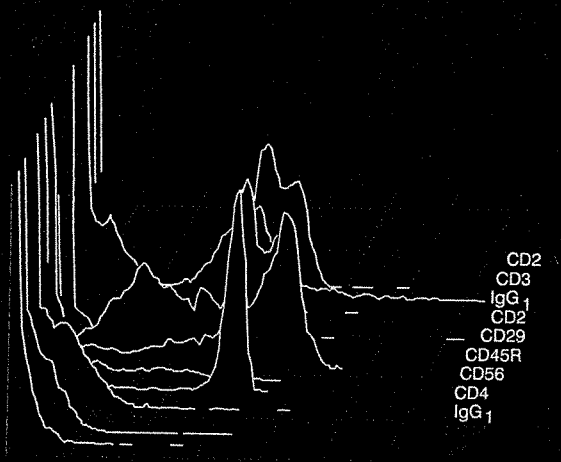

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