(g) Simultaneous Measurement of NADPH Oxidase Activity and Phagosomal Oxidation with Hydroethidine and 2',7'-Dichlorofluorescin Diacetate

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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. Hydroethidine can be oxidized to red fluorescent ethidium bromide by superoxide anion directly, whereas the oxidation of 2',7'-dichlorofluorescin to green fluorescent 2',7'-dichlorofluorescein requires hydrogen peroxide and peroxidase. The simultaneous two-color analysis of hydroethidine oxidation and 2',7'-dichlorofluorescin oxidation permits the detection of selective defects of the phagosomal oxidation following lysosomal degranulation in diseases such as sepsis.

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)

hydroethidine (MW 315)
- stock solution: 10 mM in DMF (3.15 mg/ml)
- working solution: 1 mM (1:10 dilution of stock in HBSS)

2',7'-dichlorofluorescin diacetate (DCFH-DA) (MW 487.3)
- stock solution: 10 mM (4.87 mg/ml) in DMF
- working solution: 1 mM (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)

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 2×10^7/ml unseparated leukocytes.
3. Put 1.00 ml HBSS, 20 µl cell suspension, and 10 µl hydroethidine working solution in a 12 x 75 mm polypropylene test tube (final hydroethidine concentration 10 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. 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).
5. 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 2',7'-dichlorofluorescein green fluorescence
600 nm longpass for ethidium bromide (viable cells) and PI (dead cells) red fluorescence

Reagents

HBSS	Sigma Chemical Co.	# H-1387
Hydroethidine	Molecular Probes	# D-1168
2',7'-Dichlorofluorescin diacetate	Serva	# 19353
Propidium iodide	Serva	# 33671
Phorbol 12-myristate 13-acetate	Sigma Chemical Co.	# P-8139

References

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NOTES:

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