(h) Measurement of NADPH Oxidase Activity with Hydroethidine

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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, in addition to oxidation by hydrogen peroxide and peroxidase. This is in contrast to substrates such as dihydrorhodamine 123 or 2',7'-dichlorofluorescin which are not oxidized by the superoxide anion. Stimulation of neutrophils with PMA or by phagocytosis of bacteria results in an 8- to 20-fold increase of cellular red fluorescence.

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)

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)

trypan blue (MW 960.8)
stock solution: 10 mM 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 x 10^7/ml unseparated leukocytes.
3. **For PMA stimulation**, 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. **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 hydroethidine working solution for 15 minutes at 37°C.
5. 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).
6. Quench dead cell fluorescence instead of counterstaining if highly active cells overlap with PI fluorescence. Add 5 µl trypan blue solution to 250 µl stained cells (final trypan blue concentration

200 μM) and run immediately.

7. Run on flow cytometer.

Excitation: 488 nm (argon laser) or high pressure mercury arc lamp with either 360-380 or 470-500 nm bandpass filter

Filters: 600 nm longpass for ethidium bromide (viable cells) and PI (dead cells) red fluorescence
optional 420-460 nm bandpass for HE blue fluorescence as a control on substrate loading

Reagents

HBSS	Sigma Chemical Co.	# H-1387
Hydroethidine	Molecular Probes	# D-1168
Propidium iodide	Serva	# 33671
Trypan blue	Serva	# 37252
Phorbol 12-myristate 13-acetate	Sigma Chemical Co.	# P-8139

References

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NOTES:

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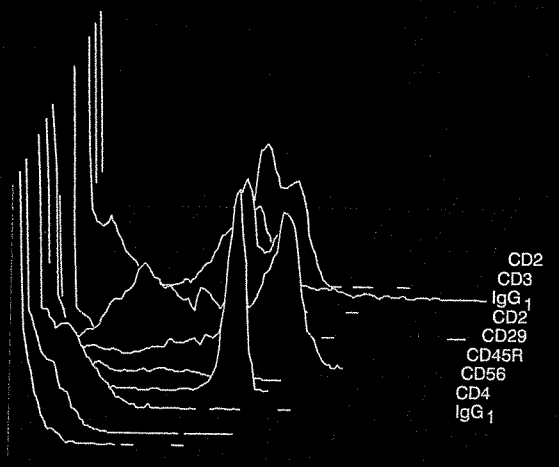

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