(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 3 mM 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

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Supplier</th>
<th>Code</th>
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<tbody>
<tr>
<td>HBSS</td>
<td>Sigma Chemical Co.</td>
<td># H-1387</td>
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<tr>
<td>Hydroethidine</td>
<td>Molecular Probes</td>
<td># D-1168</td>
</tr>
<tr>
<td>Propidium iodide</td>
<td>Serva</td>
<td># 33671</td>
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<tr>
<td>Trypan blue</td>
<td>Serva</td>
<td># 37252</td>
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<tr>
<td>Phorbol 12-myristate 13-acetate</td>
<td>Sigma Chemical Co.</td>
<td># P-8139</td>
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References


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