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Isolation of Neutrophils/Assay of O_2^- (Superoxide Anion Radical) Generation by Cytochrome-C Reduction

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Introduction

Human neutrophils play critical roles in host defence against microorganisms and in inflammatory responses. Because neutrophils in the peripheral blood of healthy individuals are not primed, stimulation-dependent responses *in vitro* are weak. Cytokines such as TNF-α and G-CSF induce a 'primed state' in neutrophils characterized by an increased capacity to produce $\cdot O_2^-$ (superoxide anion radical) adherence to endothelial cells, migration, lysosomal enzyme release, and cytocidal activity. The enhancement of neutrophil responses in this fashion has been termed 'priming'.

Here, we describe procedures for the preparation of human peripheral neutrophils and for the analysis of cytokine-induced priming of neutrophils monitored by measuring $\cdot O_2^-$ generation.

Protocol

Isolation of neutrophils

Method1-isolation of human neutrophils by Ficoll-Paque solution (Figure 1) (When the use of heparin is unfavorable Method II should be used).

1. Sample human peripheral blood (20 mL; heparinized with heparin (500-1000 units/mL Noboheparin, 0.4 mL) in a 50 mL plastic syringe).
2. Mix well and change the needle to 18 gauge (18G × 3.5).
3. Aspirate dextran1 (2% w/v, 13 mL) and mix well by inverting the synage.
4. Leave at room temperature for 20 min for sedimentation of RBCs.

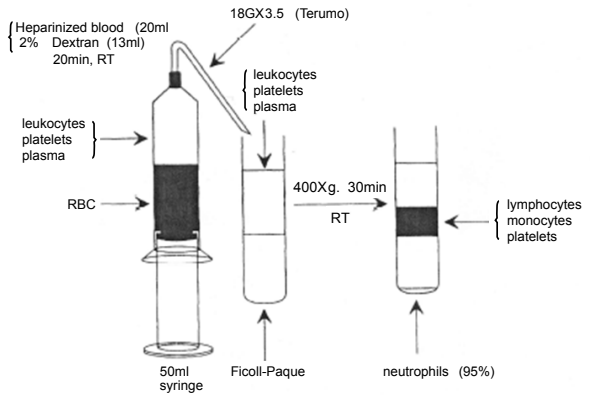

Figure 1: Isolation of human neutrophils by use of Ficoll-Paque solution.

5. Bend the syringe needle and dispense the upper layer into a 50-mL plastic centrifuge tube.
6. Carefully layer Ficoll-Paque solution (5 mL) at the bottom of the centrifuge tube.
7. Centrifuge at 1500 rpm (400 g) for 30 min at room temperature.
8. Remove the upper layer by aspiration.

[I] If many R8Cs contaminate the cells (precipitate), add NaCI (0.033 M, 50 mL). mix well with a plastic pipette, then divide cell suspension into two tubes.

[II] After 30 s add NaCI (0.27 M, 25 mL) to each tube and mix

]III] Spin at 900 rpm (150 g) for 5 min at room temperature.

[IV] Remove the upper layer by aspiration.

9. Add ice-cold Ca^{2+} -free KRP (50 mL) and re-suspend cells (as precipitate).
10. Centrifuge at 900 rpm (150 g) for 5 min at room temperature.
11. Remove the upper layer by aspiration and re-suspend cells (as precipitate) in ice-cold Ca^{2+}-free KRP (1×10^8 cells/mL). More than 95% of the obtained cells are neutrophils.

Method II: Isolation of neutrophils using mono-poly resolving medium (M-PRM) (Figure 2)

This method is for the rapid preparation of fresh neutrophils.

- Aspirate human peripheral blood (20 mL) with a 50-mL plastic syringe containing sodium citrate solution (3.8% w/v, 2 mL) and mix well.
- Carefully layer blood (5 mL) on a mixture of M-PRM solution (3.5 mL) and Ca^{2+}-free KRP (150 µL) in a 10 mL plastic centrifuge tube.
- Centrifuge at 1500 rpm (400 g) for 30 min at room temperature.
- Remove fraction 1 by Pasteur pipette.
- Transfer fraction 2 by plastic pipette to a 50-mLplastic centrifuge tube containing Ca^{2+}-free KRP (10 mL).

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- Centrifuge at 1500 rpm (400 g) for 5 min at room temperature.
- Wash twice with Ca^{2+}-free KRP (1200 rpm, 250 g, 5 min, room temperature).
- Re-suspend in ice cold Ca^{2+}-free KRP (1×10^8 cells/mL). If many RBCs contaminate the cells, treat with hypotonic solution as described in Method I.

Assay of neutrophil superoxide anion radical generation

Cytochrome-c reduction method, using a dual beam spectrophotometer equipped with a thermostatically controlled cuvette holder and a magnetic stirrer.

1. Set the dual beam spectrophotometer and recorder (37°C, stirring, absorbance $A_{550—540nm}$.
2. Add Ca^{2+}-free KRP (2 mL) containing glucose (10 mM) and cytochrome-c (20 μM) to the cuvette (cytochrome-c (2 mM, 20 μL) and glucose (1 M, 20 μL) are added to Ca^{2+}-free KRP (1.96 mL)).
3. Add neutrophils (1×10^8 cells/mL, 10 μL; final concentration 5×10^5 cells/mL.
4. After 2 min add TNF-α (2000 units/mL, 10 μL; final concentration 10 units/mL) or G-CSF (5 μg/mL, 20 μL; final concentration 50 μg/mL) or other stimuli.
5. After 10 min add FMLP, PMA or other stimuli (10 μL; final concentrations of FMLP and PMA 5×10^{-8} and 5×10^{-10} M, respectively) and record absorbance $A_{550\text{-}540\,nm}$ continuously.

Results and Calculations

The amount of $\cdot O_2^-$ generated was calculated from $\Delta A_{550\text{--}540\ nm}$/min and a millimolar absorption coefficient of 19.1 mM/cm according to the equation:

Cytochrome-c reduction (nmol/min/10^6 cells/mL) = ($\Delta A_{550\text{--}540\ nm}$/min) ×19.1×1000

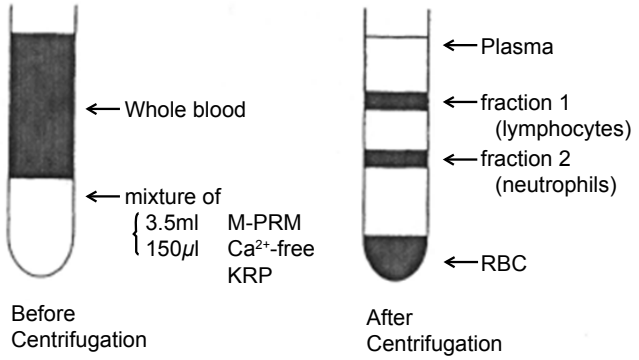

Figure 2: Isolation of neutrophils by use if mono-poly resolving medium.

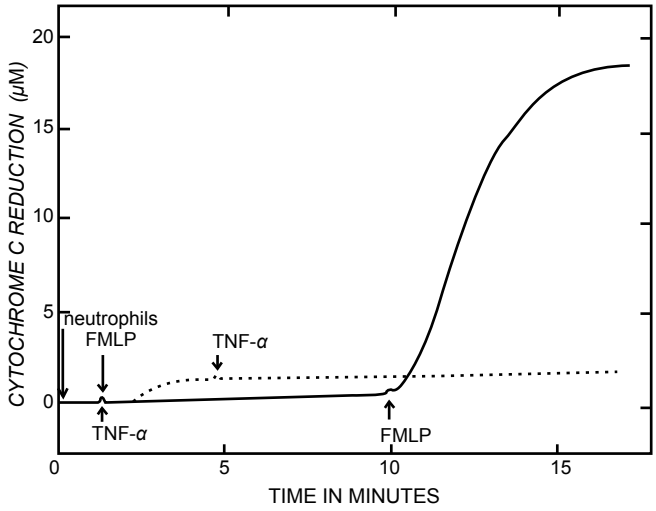

Figure 3: Enhancement of FMLP-dependent $\cdot O_2^-$ generation by TNF-α. $\cdot O_2^-$ generation was assayed by reduction of cytochrome-c as described in the text. $\cdot O_2^-$ generation was triggered by adding FMLP (1.25×10^{-8} M); TNF-α (10 units/mL) was added before or after addition of FMLP.

Because cytochrome-c can be reduced by other radical species, a control experiment using superoxide dismutase (SOD, final concentration of 15 μg/mL) should be performed to confirm that the reduction is dependent on $\cdot O_2^-$.

Human peripheral neutrophils from healthy individuals are not primed and, hence, only a low level of $\cdot O_2^-$, generation was induced by the treatment of FMLP (1.25×10^{-8} M), as shown by a dotted line in Figure 3. This weak $\cdot O_2^-$ generation was not affected by subsequent treatment with TNF-α(10 units/mL). In contrast, when neutrophils were first treated with TNF-α(10 units/mL), they underwent priming and the rate of $\cdot O_2^-$ generation induced by the treatment with FMLP (1.25×10^{-8} M) was remarkably increased, as shown by a solid line in Figure 3 [1,2].

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