A Simple Method for Isolating Human and Rabbit Polymorphonuclear Neutrophils (PMNs)

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We report the successful application to human venous blood of a novel method developed to purify rabbit polymorphonuclear neutrophils (PMNs) from whole blood. Human PMNs were separated from whole blood after sedimentation with dextran and histopaque density gradient centrifugation. $3.92 \pm 0.26 \times 10^6$ PMNs per ml of blood was harvested. The purity of the preparation was $92.00 \pm 1.10\%$. The PMNs isolated were capable of generating a high amount of reactive oxygen species (ROS) and elastase after stimulation with phorbol 12-myristate 13-acetate (PMA): $13.43 \pm 0.3 \mu\text{M}$ of O_2^- , $9.62 \pm 0.15 \mu\text{M}$ of H_2O_2 and $5.48 \pm 0.01 \mu\text{M}$ of elastase. This method gives equivalent yield and viability when applied to isolating human or rabbit PMNs, in comparison with standard methods used to isolate human PMNs. Our method could be usefully exploited for comparative studies of rabbit and human PMNs with a cellular model of inflammatory oxidative stress in which the monitoring parameters are ROS and elastase. Thus, the results of animal (rabbit) studies can be extended to human diseases.

Key words PMN; separation; reactive oxygen species; elastase; dextran sedimentation; histopaque

Pure cell populations are required to investigate the functional properties of polymorphonuclear neutrophils (PMNs). Appropriate *in vitro* methods to separate pure PMN from peripheral blood are therefore necessary, especially when animal and human peripheral blood PMNs are needed for a comparative study or to extend the results of animal studies to human diseases. However, the different PMN-lymphocyte densities (which are species-dependent), blood volume and PMN concentration, high platelet and red cell counts do not always yield satisfactory results when the blood of different species is used in a standard method.^{1,2)} In this study, we used method employed to isolate pure rabbit PMN to separate pure human PMNs from peripheral blood. This method involves sedimentation with dextran and histopaque density gradient centrifugation.³⁾ Functional responses of PMNs were investigated with the release of reactive oxygen species (ROS) and elastase by phorbol 12-myristate 13-acetate-stimulated (PMA-stimulated) PMNs. PMA is known to activate protein kinase C (PKC) directly to stimulate different oxygen species.⁴⁾ Moreover, it also induces the production of elastase.⁵⁾ Finally, we compared purity, viability, yield, ROS and elastase production using this method (for rabbit and human blood) with results obtained using a human standard method, Cabanis's method.⁶⁾

MATERIALS AND METHODS

Drugs and Chemical Products Buffer compounds and chemical reagents were purchased from Sigma Chemical Company (St Louis, MO, U.S.A.).

Isolation of Neutrophils. Sedimentation with Dextran and Histopaque Density Gradient Centrifugation Ten milliliters of fresh heparinized blood was diluted with 30 ml of phosphate-buffered saline 0.1 M pH 7.4 (PBS). Then, 8 ml of 6% dextran (6% in NaCl 0.9%) was added as previously described.³⁾ After red cell sedimentation, the supernatant was haemolyzed for 3 min. Following centrifugation (400 g, 10 min), the white cell pellet was washed and resuspended in 10 ml of PBS. Ten ml of Histopaque[®] was added at the bottom

of the tube and the PMN pellet was collected after centrifugation (400 g, 30 min). These PMNs were resuspended in hanks buffer saline solution pH 7.4 (HBSS). The concentration of the suspension was established using a Thoma counting chamber. PMN purity was checked by May-Grünwald-Giemsa staining. Viability was checked by the trypan blue exclusion test.

Cabanis's Method⁶⁾ Neutrophils were purified from fresh heparinized venous blood of healthy human subjects. Briefly, neutrophils were isolated by a density gradient technique; cells isolated by this technique were always >95% viable as determined by trypan blue exclusion, and 95% of them were neutrophils. Cells were suspended in HBSS.

Assessment of Reactive Oxygen Species Produced and Release of Elastase. Measurement of O_2^- PMNs stimulated by PMA were measured by reducing ferricytochrome C.⁷⁾ The slope of the absorbance curve at 550 nm was converted to micromoles of O_2^- using the extinction coefficient

$$E_{550} = 21.1 \text{ mm} \cdot \text{cm}^{-1}$$

Measurement of H_2O_2 The rate of hydrogen peroxide (H_2O_2) produced by PMA-stimulated PMNs was determined by the peroxidase-dependent oxidation of phenol red, as previously reported.⁸⁾ Optical density (OD) of cell-free supernatants was measured at 610 nm following the addition of 50 μl of 1 N NaOH. The slope of the absorbance curve at 610 nm was converted to micromoles of H_2O_2 using the equation:

$$\text{H}_2\text{O}_2 (\mu\text{M}) = (\text{OD} + 1.52 \times 10^{-3}) / 1.29 \times 10^{-2}$$

Human Elastase Released PMNs ($2 \times 10^6 \text{ ml}^{-1}$) were stimulated with PMA (160 nM) at 37°C. Cell-free supernatant was assayed for elastase activity. Elastase concentration was based on a standard curve using varying concentrations of reagent grade elastase, as previously described.⁹⁾

RESULTS AND DISCUSSION

As PMNs are major host defence components against microbial invasion,¹⁰⁾ it is important to study their functions.

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Table 1. Comparison of Different Methods for the Purification of PMNs

Parameters of comparison	Method of sedimentation with dextran and histopaque density gradient centrifugation		Cabanis's method
	Human PMNs	Rabbit PMNs	Human PMNs
Blood quantity (ml)	10	10	15
Preparation time (min)	70	70	65
Lymphocytes (%)	5	4	2
Million PMNs per ml of blood	3.92±0.26	2.66±0.22	3.8±0.50
Million erythrocytes per million PMNs	0.4±0.45	0.5±0.20	0.3±0.10
Viability (%)	96.00±0.20	93.50±1.50	97.50±1.00
ROS and elastase released by PMA-stimulated PMNs			
O ₂ ⁻ (μM)	13.43±0.31	10.51±0.80	13.34±0.50
H ₂ O ₂ (μM)	9.62±0.15	10.00±0.50	12.40±0.18
Elastase (μM)	5.77±0.01	2.66±0.32	6.51±0.47

Effective methods are required to isolate PMNs for such investigation; however, standard methods developed to obtain human PMN are not satisfactory when used to isolate other PMNs species.¹⁾ Here, we report an easy method to separate rabbit as well as human PMNs. With this method $3.92 \pm 0.26 \times 10^6$ PMNs per ml of human blood and $2.66 \pm 0.22 \times 10^6$ PMNs per ml of rabbit blood can be respectively separated. Their viability as assayed by the trypan blue test was: $96 \pm 0.20\%$ for human PMNs and 93.5 ± 1.50 for rabbit PMNs; corresponding purity was $92 \pm 1.1\%$ and $91 \pm 1\%$. These PMNs developed a functional response monitored in terms of their ability to mount a respiratory burst which was evaluated as ROS, and elastase generated by PMA stimulated PMNs. The amount of ROS per million PMNs was $13.43 \pm 0.31 \mu\text{M}$ (human cells) and $10.51 \pm 0.80\%$ (rabbit cells) for O₂⁻; $9.62 \pm 0.15 \mu\text{M}$ (human cells) and 10.00 ± 0.50 (rabbit cells) for H₂O₂. For elastase the amount generated was, respectively, $5.77 \pm 0.01 \mu\text{M}$ (for human PMNs) and $2.66 \pm 0.32 \mu\text{M}$ (for rabbit PMNs).

The most frequently used processes among human standard methods to isolate PMNs are: Boyum, Cabanis's and Eggleton's methods. Respectively, $2.1 \pm 0.50 \times 10^6$, $3.8 \pm 0.50 \times 10^6$ and $3.5 \pm 0.60 \times 10^6$ PMNs per ml of human blood can be separated with these methods. Viability of these PMNs are always identical with those of Cabanis's method.⁶⁾ Boyum method is time-consuming (95 min for preparation) and gives a low yield of PMNs. Eggleton's method gave PMNs which produced 40% less H₂O₂ because of erythrocyte contamination as compared with Cabanis's PMNs. However, none of these three methods is appropriate to isolate rabbit PMNs.³⁾ Comparison showed that the Cabanis technique appeared to be more practical. The three processes shown in Table 1 provide PMNs separated at the same time using blood from healthy donors; their yield and viability were almost identical. Human PMNs isolated by our method gave a similar response to PMA-stimulus as compared to PMNs isolated by Cabanis's method. However, to really compare the effects of drug on the rabbit and human PMN oxidative metabolism or generating elastase we must used the same PMN isolation means for the two groups. Unfortunately, the Cabanis method failed to isolate rabbit PMNs. Obviously, this as well as the main human standard method did not yield satisfactory results with rabbit blood because of high contamination by lymphocytes and erythrocytes, low

isolation yield and cellular viability.³⁾ Moreover, the exposure time to ammonium chloride is too long (15 min instead of the 3 min used in our method) and this substance has some disadvantageous effects on PMN function.¹¹⁾

Furthermore, ROS and elastase play an important role in the pathogenesis of inflammatory disorders. On the one hand, elastase levels are an important marker of the extent and severity process.¹²⁾ On the other hand, ROS are regarded as bioeffector molecules.¹³⁾ Thus, reproducing the conditions to evaluate ROS and elastase generation are important for the pathogenesis of inflammation studies.

Finally, our method which can rapidly provide (human or rabbit) PMNs with high viability and purity can be an interesting tool for inflammation investigation.¹⁴⁾ It can be particularly used for *in vitro* comparative pharmacological studies of drugs in both cellular species models of inflammatory stress (ROS and elastase as parameters).

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