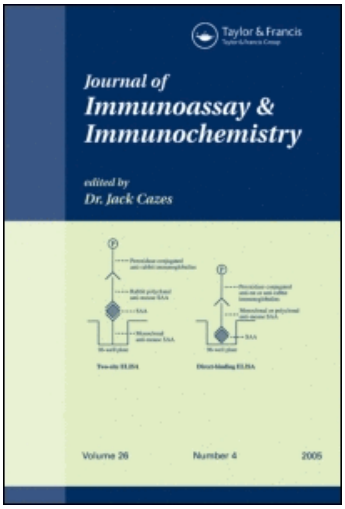

Journal of Immunoassay and Immunochemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

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To cite this Article De, Asit K. , Roach, Sarah E. , De, Mita , Minielly, Robin C. , Laudanski, Krzysztof , Miller-Graziano, Carol L. and Bankey, Paul E.(2005) 'Development of a Simple Method for Rapid Isolation of Polymorphonuclear Leukocytes from Human Blood', Journal of Immunoassay and Immunochemistry, 26: 1, 35 — 42
To link to this Article: DOI: 10.1081/IAS-200041157
URL: http://dx.doi.org/10.1081/IAS-200041157

Journal of Immunoassay & Immunochemistry, 26: 35–42, 2005

ISSN 1532-1819 print/1532-4230 online
DOI: 10.1081/IAS-200041157

Development of a Simple Method for Rapid Isolation of Polymorphonuclear Leukocytes from Human Blood

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Abstract: Polymorphonuclear leukocytes (PMNs; commonly known as neutrophils) play essential roles in innate immunity and inflammation. Although there are standardized methods for the isolation of human neutrophils, they are time consuming and demand considerable technical expertise, making them unfeasible for many clinical applications. Here, we describe a simple and time-efficient technique for the isolation of human neutrophils, which adapts a readily available commercial cell preparation tube (CPT) currently in use for isolation of peripheral blood mononuclear cells (PBMC) and plasma and is now adapted to also yield neutrophils. The total time required for neutrophil isolation was less than 1 hr. Neutrophils isolated by this method were highly purified ($\geq$97%) as assessed by surface expression of the neutrophil specific marker, CD66b. Neutrophils isolated by this method were functional as demonstrated by their ability to secrete interleukin-1 receptor antagonist (IL-1RA). Neutrophils isolated using this new technique secreted significant amounts of soluble IL-1RA ($929.3 \pm 197\,pg/10^6$ cells/mL) in response to lipopolysaccharide (LPS). Use of this adapted CPT method allows simultaneous isolation of functional human neutrophils as well as PBMC and plasma. Adoption of this new method will allow the conduct of different neutrophil assays at any clinical site without requiring trained laboratory personnel or a large staff time commitment.

Keywords: Human blood, Polymorphonuclear leukocyte, Cell preparation tube

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INTRODUCTION

Human polymorphonuclear leukocytes (PMNs or neutrophils) are the first line of defense against invading microorganisms, eliminating pathogens primarily by phagocytosis.[1] Neutrophils are also known to play a critical role in various types of inflammation and cancer.[2,3] In spite of their significant role in human inflammatory responses, studying neutrophil function in the clinical setting is problematic because no simple method for their rapid isolation has been available. The most common method for human neutrophil isolation is centrifugation of peripheral blood over a Ficoll-hypaque gradient, followed by sedimentation through dextran and then lysis of residual erythrocytes.[4] This method is time consuming and requires trained laboratory personnel to produce and maintain the gradient between the peripheral blood and the Ficoll-hypaque solution, making the isolation procedure, in general, difficult. A further complication in human neutrophil studies is their rapid apoptosis.[2] A rapid neutrophil isolation technique has the added benefit of producing physiologically intact (not undergoing apoptosis) neutrophils. BD Biosciences Corp. (San Jose, CA) has introduced a cell preparation tube (CPT) that contains a gel lock to maintain the gradient between the Ficoll-hypaque and the blood, thereby making the isolation of peripheral blood mononuclear cells (PBMC) feasible for clinical studies.[5–8]

Recently, a combination of commercially available rosettesep antibody cocktail (StemCell Technologies, Vancouver, BC) and use of CPT has made even the isolation of individual cells such as monocytes or T cells more simple and rapid. However, no such simple and rapid method was available for isolation of neutrophils. Here, we have developed a simple method for rapid isolation of functional neutrophils using commercially available CPT.

EXPERIMENTAL

Materials

CPT containing sodium heparin or sodium citrate were purchased from BD Biosciences. Bacterial lipopolysaccharide (LPS; *Escherichia coli* 0111:B4) was purchased from Calbiochem (San Diego, CA). The neutrophil specific surface marker antibody—anti-CD66b-FITC and an isotype control were purchased from Immunotech Coulter (Miami, FL). Interleukin-1 receptor antagonist (IL-1RA) ELISA kit was purchased from BioSource International (Camarillo, CA). Erythrocyte lysis buffer (EL buffer) was purchased from Qiagen (Valencia, CA).

Blood was collected from five normal volunteers for isolation of neutrophils. Informed consent was obtained from each subject before the blood draw and the study with neutrophils was approved by the Institutional Review Board.

Neutrophil Isolation Procedure

Peripheral blood was directly drawn into 8 mL Vacutainer CPTs containing sodium heparin or sodium citrate. The tubes were centrifuged for 25 min at 1700*g* at room temperature (RT). The plasma and the PBMC that were separated above the gel lock were discarded. The upper portion of the gel was washed twice with ice cold phosphate buffered saline (PBS; 5 mL for each wash). A 10 mL syringe was attached to an 18 gauge 1.5 in. needle and then pierced through the gel to collect the erythrocyte/neutrophil mixture into the syringe. The needle was removed and the cells were collected in a 50 mL conical tube. The cells were washed once with ice cold PBS containing 2% fetal bovine serum (FBS) by centrifugation for 10 min at 400 *g* at 4°C. The erythrocytes were lysed by treatment with 10 mL of commercially available EL buffer for 10 min at RT with intermittent vortexing of the tube. The cells were washed with ice cold PBS containing 2% FBS by centrifugation for 10 min at 400*g* at 4°C. Cells were then suspended in RPMI 1640 medium containing 10% FBS, 50 U/mL penicillin G, 50 μg/mL streptomycin, 50 μg/mL gentamycin, 2.5 μg/mL fungizone, 4 mmol/L L-glutamine, and 1% of MEM nonessential amino acids (complete medium), counted and checked for viability by trypan blue exclusion.

Neutrophil Culture

The neutrophils were cultured (1×10^6 cells/1 mL/well) in complete medium alone or in presence of LPS (100 ng/mL) for 18 hr at 37°C and 5% CO_2 atmosphere. The culture supernatants were harvested and stored at −80°C until the day of assay for IL-1RA.

Flow Cytometry

A total of 1×10^5 cells were first treated with 1 μg of human IgG for 20 min at 4°C followed by incubation with anti-CD66b-FITC labeled antibody or isotype control, for 30 min at 4°C. Cells were washed twice in PBS containing 2% FBS and resuspended in 500 μL of the same wash buffer and then analyzed on a BD FACSCalibur flow cytometer. Data are presented as the mean percentage positive cells.

ELISA

Soluble IL-1RA was quantitated in the culture supernatants by an ELISA according to the manufacturer's guidelines. The lower limit of detection for the assay was 31.3 pg/mL.

Data Analysis

Data are expressed as mean $\pm$ SEM. Student's t test was performed to compare between the two groups of data set using the Statview software. Statistical significance was considered when the p value was <0.05.

RESULTS

Cell Recovery and Viability

We first determined the recovery of neutrophils isolated using the sodium heparin containing CPT. Figure 1 shows that 8 mL of peripheral blood yielded a mean of 25.5×10^6 neutrophils (3.2×10^6 neutrophils/mL of peripheral blood), which is slightly higher than the expected recovery range ($2-3 \times 10^6$/mL), when isolated with the conventional method[9] and our own experience. Since sodium citrate (instead of heparin) is also often used as an anti-coagulant for blood drawing, we also tested whether there was

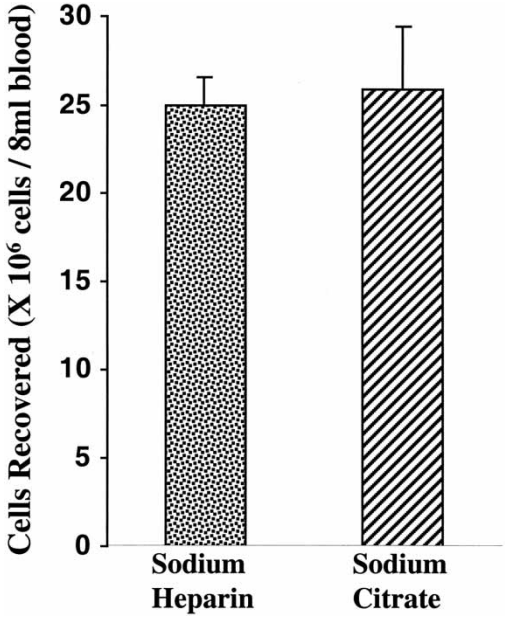

Figure 1. Recovery of human neutrophils isolated using CPTs. Eight milliliters of peripheral blood from healthy volunteers was directly collected into CPTs containing sodium heparin ($n = 5$) or sodium citrate ($n = 3$). Neutrophils were isolated as described in methods and then counted using a hemocytometer.

any difference in the yield of neutrophils isolated using these two different anti-coagulants. There was no significant difference in the recovery of cells isolated using these two different anti-coagulants (Figure 1). We also determined the viability of neutrophils by trypan blue dye exclusion. The viability of neutrophils isolated using either of the anti-coagulants was always $\geq$98%.

Cell Purity

Although neutrophils isolated by our method yielded high recovery and viability, testing the purity of the isolated cell population is an important prerequisite for their use in functional assays. Therefore, we assessed the purity of the isolated cell population by flow cytometry using an antibody against the neutrophil specific surface marker, CD66b. As can be seen in Figure 2, cells isolated using either anti-coagulant CPT type yielded $\geq$97% neutrophils.

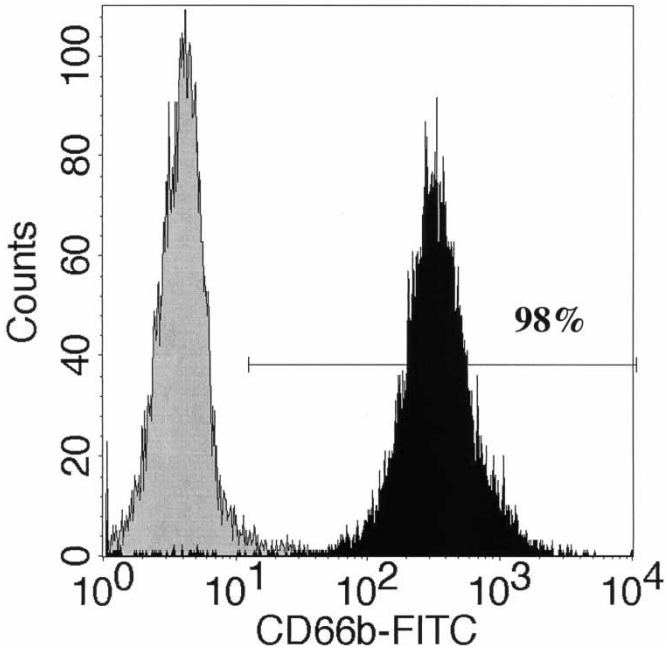

Figure 2. Assessment of purity of isolated neutrophils. Cells (1×10^6 cells/tube) were labeled with anti-CD66b-FITC antibody or matched isotype control and then analyzed on a BD FACS Calibur flow cytometer. The light shaded curve represents isotype control and the dark shaded curve represents the specific antibody data. Data are representative of five experiments with sodium heparin CPTs and three experiments with sodium citrate CPTs.

Functional Status

Finally, we tested whether the highly purified neutrophils isolated using the CPTs were functional. Human neutrophils isolated in the traditional/dextran method secrete significant amounts of IL-1RA in response to LPS.[10,11] Therefore, we assessed whether the neutrophils isolated by our novel CPT method were functionally active by the measurement of IL-1RA induction in response to LPS. As seen in Figure 3, neutrophils isolated using sodium heparin CPTs produced measurable quantities of IL-1RA (233.5 ± 28.9; $n = 4$) when cultured in medium alone. The IL-1RA production of these neutrophils was significantly increased when the cells were cultured in presence of 100 ng/mL of LPS (929.3 ± 197; $n = 4$). The neutrophils isolated using sodium citrate CPTs, produced similar levels of IL-1RA as compared to cells isolated using sodium heparin CPTs (Figure 3). These data suggest that the neutrophils isolated using both types of commercially available CPTs, maintained their functional status.

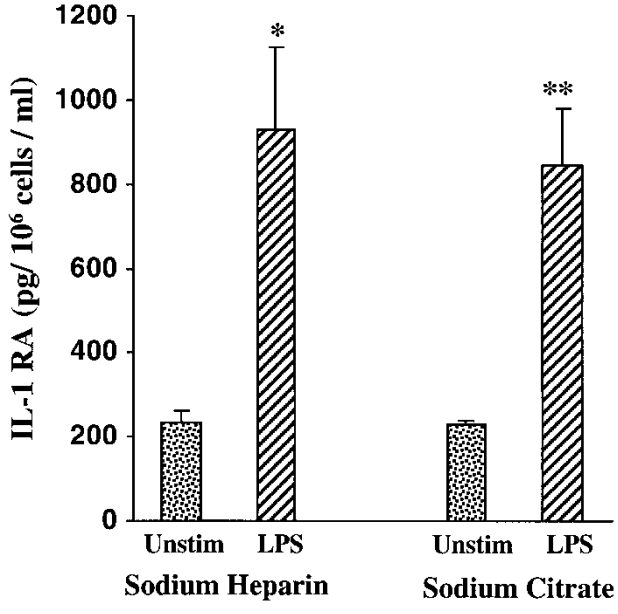

Figure 3. IL-1RA production by neutrophils. Neutrophils isolated using CPTs containing sodium heparin ($n = 4$) or sodium citrate ($n = 3$) were cultured for 18 hr in medium alone or in presence of LPS (100 ng/mL). The culture supernates were harvested and tested for IL-1RA levels by an ELISA. Data are presented as mean $\pm$ SEM. $^{*}p = 0.008$ and $^{**}p = 0.01$ as compared to unstimulated group.

DISCUSSION

Current isolation of human neutrophils from peripheral blood is both technically demanding and time consuming. This makes studying neutrophil function quite difficult in the clinical setting to implement. The major goal of this study was to develop a simple and rapid method for isolation of viable and functional human blood neutrophils. Our new method uses the commercially available CPT tubes (commonly used for isolation of PBMC and plasma) eliminating manual maintenance of gradients between Ficoll-hypaque and blood by introduction of a gel lock system. This method thus provides the added advantage of minimizing separation failure. This new method will provide simultaneous rapid isolation of plasma, PBMC, and neutrophil from the same blood sample.

Functionally active neutrophils produce cytokines and cytokine inhibitors.[10,11] However, cytokine production by purified human neutrophils is minimal ($<1.5\%$) when compared with PBMC. In contrast, production of cytokine inhibitors such as IL-1RA is fairly significant (about 15.5% of the quantity produced by PBMC).[10] Therefore, the assessment of neutrophil IL-1RA production, rather than the measurement of proinflammatory cytokine production may be more appropriate to assess the neutrophil functional status after isolation by this technique. The neutrophils isolated following our new method maintained functionally active status in in vitro culture as demonstrated by their ability to secrete significant amounts of IL-1RA in response to LPS.[9,10]

In conclusion, we have developed a new simple method for rapid isolation of functionally active human neutrophils. This novel method will be most useful for combining neutrophil studies on plasma and/or PBMC in clinical settings, where lack of time and trained laboratory personnel are major issues.

ABBREVIATIONS

PMN, polymorphonuclear leukocytes; CPT, cell preparation tube; IL-1RA, interleukin-1 receptor antagonist; ELISA, enzyme linked immunosorbent assay; PBS, phosphate buffered saline; FBS, fetal bovine serum; PBMC, peripheral blood mononuclear cell; EL, erythrocyte lysis

ACKNOWLEDGMENTS

This work was supported by the funds provided by the Department of Surgery, University of Rochester. We also sincerely acknowledge the investigators of the "Inflammation and the Host Response to Injury" Large Scale Collaborative Research Program (U54 GM-62119-03) for providing the intellectual challenge of developing a simple and rapid isolation method for human neutrophils in a clinical setting.

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Received June 30, 2004
Accepted July 31, 2004
Manuscript 3139