Video Article

Fast and Specific Assessment of the Halogenating Peroxidase Activity in Leukocyte-enriched Blood Samples

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Abstract

In this paper a protocol for the quick and standardized enrichment of leukocytes from small whole blood samples is described. This procedure is based on the hypotonic lysis of erythrocytes and can be applied to human samples as well as to blood of non-human origin. The small initial sample volume of about 50 to 100 µl makes this method applicable to recurrent blood sampling from small laboratory animals. Moreover, leukocyte enrichment is achieved within minutes and with low material efforts regarding chemicals and instrumentation, making this method applicable in multiple laboratory environments.

Standardized purification of leukocytes is combined with a highly selective staining method to evaluate halogenating peroxidase activity of the heme peroxidases, myeloperoxidase (MPO) and eosinophil peroxidase (EPO), *i.e.,* the formation of hypochlorous and hypobromous acid (HOCl and HOBr). While MPO is strongly expressed in neutrophils, the most abundant immune cell type in human blood as well as in monocytes, the related enzyme EPO is exclusively expressed in eosinophils. The halogenating activity of these enzymes is addressed by using the almost HOCl- and HOBr-specific dye aminophenyl fluorescein (APF) and the primary peroxidase substrate hydrogen peroxide. Upon subsequent flow cytometry analysis all peroxidase-positive cells (neutrophils, monocytes, eosinophils) are distinguishable and their halogenating peroxidase activity can be quantified. Since APF staining may be combined with the application of cell surface markers, this protocol can be extended to specifically address leukocyte sub-fractions. The method is applicable to detect HOCl and HOBr production both in human and in rodent leukocytes.

Given the widely and diversely discussed immunological role of these enzymatic products in chronic inflammatory diseases, this protocol may contribute to a better understanding of the immunological relevance of leukocyte-derived heme peroxidases.

Video Link

The video component of this article can be found at http://www.jove.com/video/54484/

Introduction

Polymorphonuclear leukocytes (PMNs, also called granulocytes) and monocytes represent important cellular components of the innate immune system in the blood[1,2]. They contribute to the primary defense against pathogens as well as to the activation of the acquired immune system and the initiation of a systemic inflammatory response[2-4]. Yet especially neutrophils, the most abundant type of granulocytes, and monocytes also significantly contribute to the regulation and termination of acute inflammatory events[5]. Therefore these cells may also play an important role in chronic inflammatory diseases like rheumatoid arthritis[6,7]. In fact, asthma, a chronic inflammatory airway disease, is characterized by an impaired apoptosis of eosinophils, the second most granulocyte type in the blood[8]. Yet the apoptosis of granulocytes and their quick removal by macrophages are two essential steps during the cellular termination of inflammation[9-11].

In the named immune cells two closely related enzymes, namely myeloperoxidase (MPO, neutrophils and monocytes) and eosinophil peroxidase (EPO, eosinophils) can be found[12,13]. These heme peroxidases are classically related to the humoral immune response as they two-electronically oxidize (pseudo-)halides to the corresponding hypo(pseudo)halous acids which are known for their bactericidal properties[14-16]. Under physiological conditions MPO mainly forms hypochlorous acid (HOCl) and hypothiocyanite ($^{-}$OSCN) while the latter and hypobromous acid (HOBr) are formed by EPO[17-19]. New results suggest that this (pseudo-)halogenating enzyme activity may also contribute to the regulation of inflammatory responses and to the termination of immune reactions[20,21]. In fact, the HOCl production by MPO and derived products were shown to suppress T cell-based adaptive immune responses[22-24].

In order to gain more insights into the immunological role of leukocytes from the innate immune system at chronic inflammatory diseases and to determine the contribution of MPO and EPO to this physiological function we developed a method to quickly enrich leukocytes from small blood samples for a subsequent specific determination of the halogenating peroxidase activity in these cells. For erythrocyte depletion we have chosen

a standardized method including two-subsequent hypotonic lysis steps with distilled water, which leads to a quick leukocyte enrichment at low material costs. For the subsequent determination of the halogenating MPO and EPO activity the HOCl- and HOBr-specific dye aminophenyl fluorescein (APF) was used[25-27]. In contrast to the application of unspecific peroxidase staining methods[28,29], this approach allows the selective detection of the halogenating peroxidase activity, which is often impaired at severe inflammation[30,31].

Protocol

All human blood samples were obtained from healthy volunteers, and the applied leukocyte enrichment protocol follows the guidelines of ethics commission of the Medical Faculty of the University of Leipzig. The experiments with rat blood were approved by the responsible local ethical committee (Landesdirektion Sachsen, Referat 24), according to the German guidelines on animal care and use.

1. Experimental Setup

NOTE: As the hypotonic lysis procedure for the depletion of erythrocytes from the blood samples is a time-critical procedure, prepare all necessary equipment (*e.g.,* buffers) for this part of the protocol in advance.

1. Label one 15 ml centrifuge tube for hypotonic lysis and one 1.5 ml sample tube for subsequent aminophenyl fluorescein (APF) staining per blood sample.
    1. If the leukocyte enrichment will be performed under sterile conditions, perform this labeling under a flow box.

2. Prepare about 12 ml phosphate-buffered saline (PBS, 10 mM) per sample. Depending on whether the leukocytes shall be isolated under sterile conditions or not, prepare PBS either by using sterile ready solution from a supplier or by dissolving PBS tablets in distilled water. Check the pH value and, if necessary, adjust to pH 7.4 by using small amounts of 0.1 M HCl and NaOH solutions.
    1. Dissolve PBS tablets (see Table of Materials) in 200 ml distilled water to obtain a non-sterile buffer solution sufficient for about 16 samples. If desired, sterilize this solution by sterile filtration.

3. Prepare about 1 ml Hanks balanced salt solution (HBSS) supplemented with Ca^{2+} per sample.
    1. Dissolve 970 mg of HBSS salt in 100 ml (final volume) double-distilled water. Before filling up to the final volume, check the pH value and adjust it to pH 7.4. Due to its low buffer capacity, prepare this buffer freshly each day and perform the pH adjustment with special care by using small amounts of 0.1 M HCl and NaOH solutions.
    NOTE: Sterile solution may be used, depending on the experiment. The solution may be sterilized by sterile filtration.

4. Besides the two buffers, label and prepare a tube (*e.g.,* 50 ml centrifugation tube) or flask (*e.g.,* 250 ml measuring cup) with double-distilled water for the hypotonic lysis procedure in advance. Prepare about 10 ml water per sample.
5. Prepare a container with crushed ice for the APF staining (section 3), which is performed on ice.
6. Prepare aliquots of APF in advance to allow the quick preparation of working solutions used during the staining (step 3.2) and to avoid repeatedly freezing and thawing of the APF solution.
    NOTE: APF is commonly obtained as a 5 mg/ml (11.81 mM) stock solution in methyl acetate.
    1. Freeze aliquots of 10-100 µl in 0.5 ml tubes. For the APF staining a final dye concentration of 10 µM is used (step 3.8).

2. Hypotonic Lysis of the Erythrocytes

NOTE: Perform the hypotonic lysis steps (except the centrifugation steps) under a laminar flow bench to avoid contamination. Perform the whole procedure at room temperature. As the hypotonic lysis is a time critical process, adjust the pipettes for water (2 ml) and PBS (5 ml) addition in advance and open the water and PBS flasks before starting the procedure. Store the PBS solution and the distilled water at room temperature before usage.

1. From each blood sample transfer 100 µl to the appropriate 15 ml centrifugation tube.
    NOTE: In our experiments blood samples usually contain 10 U/ml of heparin in order to avoid coagulation. Yet due to different sources of the sample material the nature and concentration of the anti-coagulant may differ. Its influence on the APF staining should be tested by comparing the results to results obtained from blood samples supplemented with other types or concentrations of anti-coagulant.
2. Add 2 ml distilled water and mix the samples by using a vortex mixer set to a medium level. Incubate the samples for 60 sec, then add 5 ml PBS per sample and mix using the vortex mixer.
    NOTE: Up to about 5 samples can be prepared in parallel. Keep the sample order the same for the addition of water and PBS to achieve comparable incubation times.
3. After regeneration of isotonic conditions by PBS addition (step 2.2) pellet the remaining intact cells in all samples by centrifugation for 6 min at room temperature and 450 x g.
4. Remove the supernatant by pouring it into a table waste bin. Remove as much liquid as possible by inverting the centrifugation tube and tapping the opening onto a paper towel. Ensure that the cell pellet is as dry as possible.
5. Repeat the hypotonic lysis procedure as described before (step 2.2).
    NOTE: In principle this hypotonic lysis procedure (steps 2.3 to 2.5) could be repeated more than once, depending on the purity of the mixed leukocyte fraction to be obtained. After two lysis steps the share of remaining erythrocytes in the obtained mixed cell fraction is about 25%.
6. Pellet the remaining intact cells by centrifugation for 6 min at room temperature and 450 x g. Remove the supernatant (see step 2.4). Add 500 µl HBSS to the pellet and gently dissolve it until a homogenous solution is obtained. Transfer each sample to the accordingly labeled 1.5 ml sample tube.
7. Depending on the experiment directly analyze obtained samples (*e.g.,* via flow cytometry)[25], stain with fluorescence-labeled antibodies (for identification of single cell types)[32], incubate with cell stimuli (for cell activation experiments)[33] or stored on ice and use later. Depending on

the aim of the study, immediately perform subsequent experiments on the mixed leukocyte fraction since granulocytes have a fairly short half-life of about 20 hr.
NOTE: This also holds for the peroxidase activity staining described below.

3. Halogenating Peroxidase Activity Staining

NOTE: HOCl- and HOBr-production by the blood-derived heme peroxidases MPO and EPO is quantified by using APF, which is oxidized to fluorescein by the named hypohalous acids. Therefore if cell labeling with fluorescence-labeled antibodies is performed in combination with APF staining, avoid fluorophores that interfere with the emission signal of fluorescein.

1. Perform APF staining at 4 °C on ice.
2. For preparing a working solution of APF thaw an appropriate aliquot of the dye (*e.g.,* 10 µl, 11.81 mM) and dilute it with HBSS to exactly 1 mM (*e.g.,* add 108.1 µl buffer to the 10 µl).
    1. For each sample (500 µl cell solution) prepare 5 µl of the described APF working solution. Accordingly, use one 10 µl aliquot of APF (11.81 mM), which yields 118.1 µl APF working solution (1 mM) to prepare about 20 samples. Due to loss during pipetting prepare about 10% more APF working solution than calculated.

3. In order to check the peroxidase specificity of the APF staining, prepare control samples with the heme peroxidase inhibitor 4-aminobenzoic acid hydrazide (4-ABAH)[35].
    1. Prepare a 1 M stock solution of 4-ABAH (151.17 g/mol) in dimethyl sulfoxide (DMSO) by diluting 75.6 mg 4-ABAH in 500 µl solvent. Further dilute 4-ABAH 1/10 in HBSS.

4. For the preparation of a H_2O_2 working solution label two 1.5 ml centrifugation tubes with "70 mM H_2O_2" and "7 mM H_2O_2", respectively.
    1. In the tube labelled "70 mM H_2O_2", freshly dilute 10 µl of a 30% stock solution (8.8 mM) of H_2O_2 using 990 µl distilled water to obtain a concentration of about 88 mM. Store on ice and use within 4 hr.
    NOTE: H_2O_2 is typically delivered as a 30% stock solution by the suppliers. When stored at 4 °C, it is stable for about 3 years. Perform dilutions of H_2O_2 in distilled water to avoid decomposition. H_2O_2 dilutions could also be prepared in 0.1 M NaOH solution.
    2. For the determination of the exact H_2O_2 concentration prepare a further 1 to 10 dilution from the first H_2O_2 stock solution (about 88 mM) by adding 100 µl from this solution to 900 µl distilled water in the 1.5 ml tube labeled "7 mM H_2O_2".
    3. Record a spectrum in the near UV range (*e.g.,* 200-300 nm) by using an UV-Vis photospectrometer and use the absorbance at 240 nm (ε_{240} = 34 M^{-1} cm^{-1}) to determine the actual H_2O_2 concentration in the second H_2O_2 stock solution[34]. Ensure that the reference cuvette contains only distilled water. For the measurement use quartz cuvettes as a spectrum in the UV range is recorded.
    4. From this result, calculate the exact concentrations of both H_2O_2 stock solutions. Adjust the concentration of the first stock solution in the "70 mM H_2O_2" sample tube to exactly 70 mM by adding an appropriate amount of distilled water. Store the obtained working solution on ice and use within one hour.

5. Add 5 µl of the 4-ABAH working solution (100 mM) to the appropriate samples for a final concentration of 1 mM. Incubate the samples for 15 min at 37 °C in the incubator before APF addition.
6. Add 5 µl APF working solution (1 mM) to each 500 µl sample to obtain a final dye concentration of 10 µM. Mix the samples gently (*e.g.,* by using the vortex mixer on a medium setting) and incubate for 30 min at 37 °C.
7. Add 5 µl H_2O_2 working solution (70 mM) to each 500 µl sample for a final concentration of 700 µM. Gently mix the samples and incubate them for 60 min at 37 °C. Perform appropriate control measurements in parallel.
    NOTE: Control measurements showed that 700 µM H_2O_2 are not toxic for the cells. Nor were any significant cellular responses observed.
    NOTE: The APF staining may also be performed by omitting the addition of H_2O_2, depending on the scientific question. In the absence of hydrogen peroxide only the basal chlorinating MPO and EPO activity is determined while the addition of H_2O_2 allows the detection of the maximal HOCl and HOBr production by the cells. The latter means a significantly higher fluorescent signal.
8. For pelleting the cells, centrifuge the samples for 10 min at room temperature and 400 x g. Thoroughly remove the supernatant without destroying the pellet and add 250 µl HBSS to resuspend the cells.
    NOTE: The samples are now ready for analysis via flow cytometry[25].
9. Store the samples in the dark until analysis to avoid bleaching. After excitation at 480-490 nm detect the emission of fluorescein at about 525 nm[37].

Representative Results

As reported previously the method described above turned out to be applicable both to human and to non-human material[32]. Moreover as shown for mice with asthmatic symptoms the APF staining may be a suitable tool to detect differences in the systemic pro-inflammatory status. Therefore in a subsequent study we used this protocol to repeatedly evaluate the halogenating activity of MPO (and EPO) in female *Dark Agouti* rats with pristane-induced arthritis (PIA). A representative example of leukocyte enrichment and staining performed during this experiment is shown in **Figure 1**. About 200 µl whole blood was obtained from the retrobulbar venous plexus of the animal under anesthesia and heparinized. The erythrocyte depletion and a subsequent APF staining were performed by using 100 µl sample volume. Afterwards the sample was analyzed by flow cytometry.

As shown by plotting cell size versus cell granularity (**Figure 1A**), a strong depletion of erythrocytes from the sample was achieved. Furthermore different leukocyte types were clearly discernible, which were identified by applying fluorescence-labeled cell surface markers (not shown). Briefly the erythrocyte (CD235a-positive)/cell debris region only accounted for 22% of the events while about 48% of the events were identified as CD16-positive neutrophils. Furthermore 3% of the events were each identified as CCR3-positive eosinophils and CD14-positive monocytes and about 19% of the events accounted for CD5/CD19-positive B and T lymphocytes, respectively. The intensity distribution of the APF-derived fluorescence (**Figure 1B**) clearly allowed the discrimination of peroxidase-negative erythrocytes and lymphocytes while monocytes and eosinophils led to a moderate APF oxidation and neutrophils were strongly peroxidase-positive under the chosen experimental conditions. These results are in line with the high abundance of MPO in the latter cells as compared to the enzyme concentration in monocytes[20]. The relatively weak response of the eosinophils can be explained by the fact, that no bromide was added, which may have prompted the EPO-derived formation of HOBr. Preliminary studies on isolated eosinophils surprisingly did not show a big influence of externally added bromide on the APF oxidation by these cells. Still, oxidation of APF by the eosinophils was observed which may be explained by the introduction of small amounts of Br^- via the buffer solutions used (PBS and HBSS). Alternatively EPO may also produce small amounts of HOCl, at least under acidic conditions (*e.g.,* in phagolysosomes).

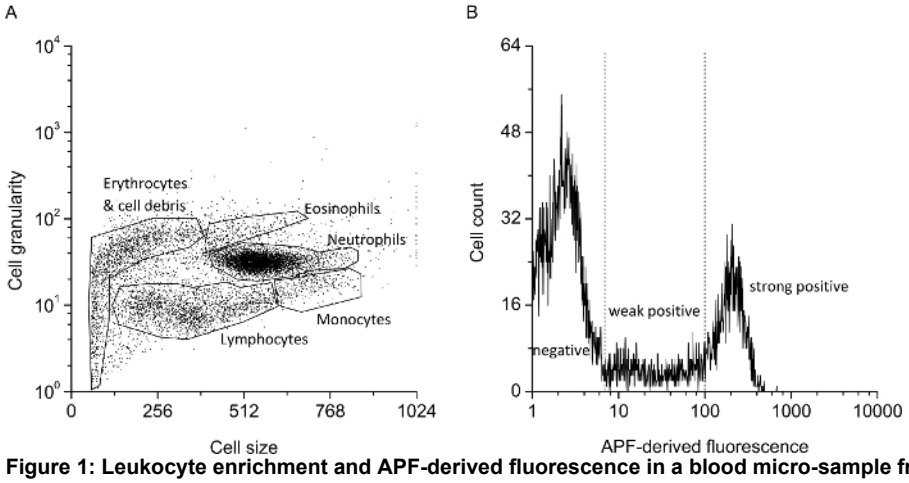

Figure 1: Leukocyte enrichment and APF-derived fluorescence in a blood micro-sample from a rat. An amount of about 200 µl whole blood was obtained from the retrobulbar venous plexus of a female Dark Agouti rat. After application of the described hemolysis procedure to 100 µl blood and a subsequent APF staining the mixed cell fraction was analyzed by flow cytometry. As shown by the FSC-/SSC- Plot (**A**) the erythrocytes were strongly depleted from the sample and the different leukocyte types could easily be distinguished. The distribution of the APF-derived fluorescence intensity (**B**) clearly showed heme peroxidase-negative cells (erythrocytes and lymphocytes) as well as leukocytes with moderate (eosinophils and monocytes) or strong (neutrophils) halogenating peroxidase activity. Please click here to view a larger version of this figure.

Discussion

As neutrophils are the most abundant leukocytes in human blood the isolation of peroxidase-positive cells often only focuses on these cells and includes a separation of neutrophils from other leukocytes by density gradient centrifugation[38]. Yet as neutrophils are much less abundant in murine blood samples[39] for the latter more complicated methods have to be used[40]. Moreover both methods also lead to the removal of peroxidase-positive monocytes from the samples and, due to the need of larger blood volumes (*e.g.,* 400 µl[41]), are not applicable to micro-samples obtained during recurrent blood sampling from small laboratory animals[38,40]. The purification of murine neutrophils from peritoneal exudates also need the sacrifice of the animals and, moreover, does not yield blood-derived granulocytes[38,40]. Recently developed methods to obtain highly purified granulocyte fractions from murine blood (*e.g.*, the application of antibodies) are often expensive, complicated and time consuming[40,42,43] and again only focus on the purification of one peroxidase-positive leukocyte type.

Thus the method presented here has a couple of advantages over other methods applied for the purification of peroxidase-positive leukocytes. As it is based on small blood samples the obtained cells represent the situation in the circulation. Due to the small initial sample volume needed for the protocol the method is applicable to both human and non-human blood, despite the species-specific differences in the abundance of neutrophils. In fact, we were even able to apply the described method to initial blood volumes as small as 50 µl (data not shown). The small blood sample needed for the method also makes it suitable for repeated blood withdrawal from small laboratory animals or for special medical applications (*e.g.,* newborn diagnosis). As the leukocyte enrichment is based on the depletion of erythrocytes all peroxidase-positive cells (neutrophils, eosinophils, monocytes) are included in the obtained mixed leukocyte fraction and can be separately analyzed by using flow cytometry. The method is fast, reliable and has low requirements regarding chemicals and instrumentation, making the protocol suitable for multiple scientific environments.

As neutrophils are easily activated one critical step during the described method is the precise adjustment of the pH value of the buffer solutions used (PBS and HBSS, see steps 1.2 and 1.3 of the protocol section). Furthermore the incubation time of the blood cells with distilled water should be no longer than 60 sec before restoring normosmotic conditions (see step 2.2 of the protocol section. The (almost) completely removal of solvent from the cell pellet (step 2.4) before the addition of water for the second hypotonic lysis is another critical step as buffer residues will diminish the efficiency of the hypotonic lysis procedure. Another critical step refers to the application of H_2O_2 during the APF staining. Although the applied amount should not be cytotoxic, cell vitality should be checked. During our studies we typically apply the dye 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimi-dazolylcarbocyanine iodide (JC-1) for the detection of early apoptotic events.

Moreover, there are a couple of limitations in the described simplified leukocyte enrichment method. While the technique can be applied to blood samples from several species (man, rat and mouse have been tried), the leukocyte amount obtained after the lysis of erythrocytes depends on their initial abundance in the blood. The latter certainly varies between species[39] and is also dependent on the inflammatory state of the probed individual. Moreover, while the lysis procedure can be done with up to one hundred samples in parallel the intermediate centrifugation steps pose a limitation as, depending on the centrifuge used, only up 30 samples can be processed in parallel. Furthermore, while APF readily detects HOCl and HOBr in the presence of SCN^-, the halogenating activity of MPO and EPO also affects to the latter pseudo-halide. Thereby hypothiocyanite (^-OSCN) is formed which is unable to oxidize APF. Another limitation comes from the properties of the APF dye used for the determination of HOCl- and HOBr-production by MPO and EPO. As the dye is oxidized to fluorescein, the staining cannot be combined with fluorescent antibodies with an emission spectrum in the same range. Of course any interferences between the APF-derived fluorescence and the signal of additional fluorescent signals have to be checked and compensated for in the flow cytometer.

Otherwise, if the detection of the halogenating peroxidase activity is not the scope of the study, after application of the erythrocyte depletion method other analytical methods may be used instead of APF. As the described hypotonic lysis procedure leads only to a partial depletion of erythrocytes and as all leukocyte are still present in the obtained mixed cell fraction, only methods which allow a single cell analysis should be applied. Methods which include the lysis of cells (*e.g.*, Western plot analysis) will not yield reliable results. The small initial sample volume may be another obstacle for such analytical methods.

Regarding the investigation of MPO (and EPO) activity in leukocytes often only the general oxidant production by the cells is addressed instead of specifically evaluating the heme peroxidase activity[44,45]. Moreover often no attempts are made to distinguish between the halogenating and the peroxidase activity of MPO and EPO[46,47]. Methods, which specifically address the chlorinating MPO activity by quantifying HOCl-derived products like chlorotyrosine do not detect over-oxidized and/or metabolized products and, thus, often lead to an underestimation of the real HOCl-production[48]. Moreover this method as well as the detection of HOCl-derived 2-chloroethidium are also time-consuming, need costly analytical equipment and include lysis of cells[48-50].There are many reports about new dyes for the specific determination of the chlorinating MPO activity within vital cells[44,51,52]. Yet to date only APF and its related compound hydroxyphenyl fluorescein (HPF) are commercially available and therefore routinely used in research[25,33].

In fact, by using APF we could show that the chlorinating MPO activity cannot only be quantified by using the isolated enzyme[53] but also by applying the dye to living cells[26,33]. Thus by combining the quick leukocyte enrichment from blood micro-samples stated above with an APF staining we developed a protocol, which is suitable to specifically and simultaneously address the halogenating activity of MPO and EPO in all peroxidase positive leukocytes, *i.e.*, neutrophils, monocytes and eosinophils[32]. Moreover the method does not require cell lysis, which allows a broad variety of analytical methods, including flow cytometry and confocal fluorescent microscopy. Moreover this peroxidase activity staining can be combined with the application of cell surface markers, which allows the specific analysis of leukocyte sub-fractions, depending on the scientific task. Yet it has to be considered that the applied fluorescence-labeled antibodies do not interfere with the fluorescein-based fluorescence signal used to quantify the HOCl- and HOBr-derived APF oxidation.

In summary this method may provide a new tool to get more insights into the physiological role of the halogenating peroxidase activity of the immunological relevant blood-derived peroxidases MPO and EPO, which is still not well understood[8,20,23]. Moreover in an animal study on rheumatoid arthritis we could recently observe that this protocol may lead to the evaluation of the MPO-derived HOCl production as a new clinical marker at chronic inflammatory diseases (unpublished data).

Disclosures

The authors have nothing to disclose.

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