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A novel methodology for NETs visualization under light microscopy

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ABSTRACT

Neutrophils are the most abundant leukocytes in the bloodstream and are very important for the resolution of infection. One of the strategies used by neutrophils to eliminate a microorganism is the formation of extracellular traps. Different methods for neutrophil extracellular traps (NETs) visualization have been described along the years, usually requiring the use of a fluorescent, confocal or scanning electron microscope. This research aimed to visualize NETs using light microscopy as another way to study NETs prior to using the more expensive techniques, making NETs research more cost effective. We evaluated neutrophil purity, viability and function by analyzing the formation of NETs comparing DAPI with safranin. When evaluating NETs formation, neutrophils that were not stimulated did not form NETs and when neutrophils were exposed to PMA or *S. aureus* NETs were formed and visualized with safranin under light microscopy and DAPI under fluorescence microscopy.

Our method demonstrates another way to visualize NETs that can be added to the standard methods of visualization of NETs, increasing the opportunities to generate knowledge in the topic in any lab around the world.

1. Introduction

Neutrophils are the most abundant leukocytes in the bloodstream and are very important for the resolution of infection. One of the strategies used by neutrophils to eliminate a microorganism is the formation of extracellular traps [1]. Neutrophil extracellular traps (NETs) are stimulated by specific cytokines (e.g., interleukin 8 (IL-8)), bacterial products (e.g., lipopolysaccharide (LPS)) and by pathogens such as *Shigella flexneri*, *Staphylococcus aureus*, *Streptococcus pneumoniae* and *Candida albicans* [2]. Stimulation of neutrophils with the diacylglycerol (DAG) mimetic phorbol 12-myristate 13-acetate (PMA) also results in the production of NETs. It is clear that NET formation following PMA stimulation is dependent on ROS production (via the NADPH oxidase system) and this is likely to follow the activation of protein kinase C (PKC) [3]. The structure of NETs released from neutrophils exposed to phorbol myristate acetate (PMA) or lipopolysaccharide (LPS) was first observed by Brinkmann et al., in 2004 using electron microscopy [1]. Since then, different methods for neutrophil visualization have been described and they often require the use of a fluorescent, confocal or scanning electron microscope plus the use of fluorescent staining and monoclonal antibodies for elastase, mieloperoxidase and histones, this equipment and reagents are hardly accessible for research sites with low budgets and/or located in low to middle income countries [4–10]. Another important factor is that when using fluorescent dyes imaging has to be done immediately to avoid fluorescence fading, this issue does not happen with simple staining dyes like safranin. Safranin is a well-known and stablished DNA dye, because of its positively-charged nature, it is a good complement for DNA's negatively charged structure [11,12]. Safranin is able to form complexes with DNA by an intercalating mechanism, with a slight preference for CG-rich segments [13,14]. This research aimed to visualize NETs using safranin under light microscopy as another way to study NETs prior to using the more expensive techniques, making NETs research more cost effective. Our method demonstrates another way to visualize NETs that can be added to the standard methods of visualization of NETs, increasing the opportunities to generate knowledge in the topic in any lab in the world.

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Abbreviations

NBT	(Nitro Blue Tetrazolium)
NETs	(Neutrophil Extracellular Traps)
PMA	(Phorbol Myristate Acetate)
ROS	(Reactive Oxygen Species)

2. Materials and methods

2.1. Study population

We recruited 5 healthy male adults between the age range of 19 and 30 at the Family Medicine Department of the University Hospital "Dr. José E. González". Subjects who were smokers and/or alcoholics, those suffering from diabetes mellitus and any other chronic illness, recent use of antibiotic or non-steroidal anti-inflammatory drugs (NSAIDS) were excluded from the study. All procedures were reviewed and approved by the Research and Ethics Committee of our institution with the number IN18-0005. All participants provided a written informed consent.

2.2. Neutrophil isolation method

Neutrophils were isolated using a methodology previously implemented in our lab [15]. Once isolated, cell viability was analyzed with trypan blue. To evaluate nuclear morphology, we implemented methylene blue staining (0.1% - 0.1 g in 100 ml of distilled water) in combination of a glass slide preparation technique previously described by Juarez-Ortega et al. [16]. Briefly, in a sterilized slide (autoclaved at 121 °C with 15 psi of pressure) with a 3 hole adherent paper, 20 μl of a cell suspension of 1×10^6/ml are placed in each circle and are incubated at 37 °C with 5% CO_2 for 30 min to allow adherence of the neutrophils. Next, the supernatant is eliminated and 20 μl of methylene blue stain 0.1% are added and the cells are incubated at room temperature for 5 min. After the 5 min the stain is washed with distilled water and the slide is air dried. Finally, the 3 circles are marked with a permanent marker and the paper is removed. 20 μl of 60% solution of synthetic resin in xylene are added in each circle as a mounting medium and a cover slide is placed on top. The slide is visualized in a optic microscope at 40x and 100x with immersion oil (Fig. 1).

2.3. Respiratory burst assay

Respiratory burst was analyzed using the method described by Mendoza-Aguilar et al., with some modifications [17]. The assay consisted of the following steps: a 5×10^6 neutrophil suspension was prepared and 100 μl (5×10^5) were added to 6 wells from a 96 well plate, 3 wells without stimulus served as negative control and 3 wells with 100 nM of PMA served as a positive control. Afterwards, 20 μl of 0.1% NBT were added to all wells. The plate was incubated for 30 min at 37 °C with 5% CO_2. Once incubation was completed, the supernatant was removed and the cell monolayers were resuspended in 200 μl of 5% SDS in 0.08 N NaOH. Finally, the plate was incubated at room temperature overnight to let the formazan crystals dissolve, and this was read at 620 nm in a spectrophotometer.

2.4. Neutrophil extracellular traps visualization

To stain NETs, 3 glass slides were prepared with a 3-hole adherent paper. One slide was designated as negative control (NS), the second one as positive control (PS) and the third one as experimental slide (ES). 20 μl of the cell suspension are added in each slide in all 3 circles (2×10^4). 10 μl of PMA (100 nM) are added to the positive control slide and *S. aureus* stained with crystal violet (2×10^5, MOI:1:10) was added to the experimental slides, all slides were incubated at 37 °C with 5% CO_2 for 1 h and 3 h. After incubation is completed, the supernatant is eliminated and the cells are fixed with 4% formaldehyde for 10 min. The supernatant is eliminated and 20 μl of 0.5% safranin stain (0.5 g of safranin in 100 ml of distilled water) or 20 μl of DAPI are added to all the circles and the cells are incubated at room temperature for 5 min. After the 5 min the stain is washed with distilled water and the slide is air dried. Finally, the 3 circles are marked with a permanent marker and the paper is removed. When using the mounting medium 20 μl of 60% solution of synthetic resin in xylene are added in each circle of the neutrophils stained with safranin and 20 μl of Vectashield are added to the neutrophils stained with DAPI and a cover slide is placed on top. The neutrophils stained with safranin are visualized in a optic microscope at 40x and 100x with immersion oil, and the neutrophils stained with DAPI are visualized in a fluorescent invertoscope (EVOS FLoid Imaging System). In order to confirm that safranin was indeed staining DNA, we decided to implement a counter stain using NBT and safranin. We followed all the steps described previously on this paragraph with the difference that in the slide stimulated with PMA we added NBT 0.1% and after 30 min and 90 min the slide was stained with safranin.

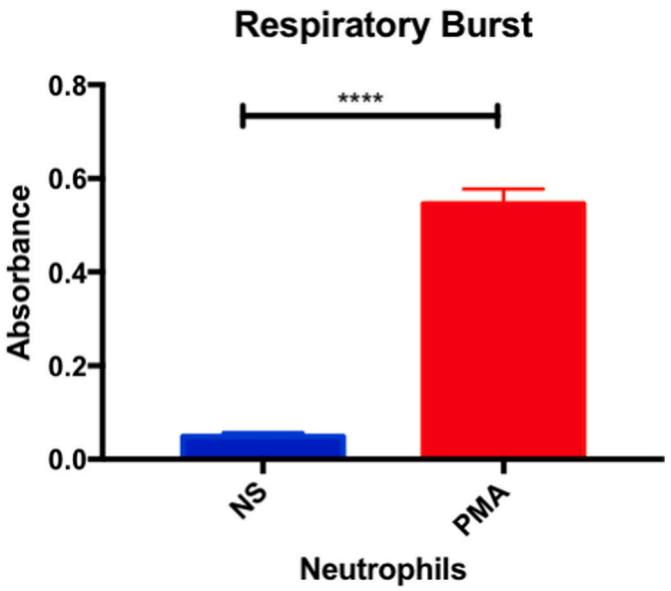

Fig. 1. Respiratory burst production in healthy donors. NBT reduction was not affected in any of the 5 donors. We obtained a mean absorbance of 0.5462 with PMA stimulated neutrophils and 0.0485 with non-stimulated neutrophils, we obtained a statistical difference $p < 0.0001$, which is expected in normal neutrophil function. Statistical analysis was performed with one-tailed paired Student's *t*-test. Bars correspond to mean values ± standard deviation (SD).

2.5. Statistical analysis

All values are expressed as mean ± SD. The results were analyzed with the GraphPad Prism software Version 7 using a one-tailed unpaired Student's *t*-test. Statistical significance was defined as $p < 0.05$.

3. Results

3.1. Isolation of neutrophils

Neutrophils were obtained from 5 healthy subjects and we obtained an average cell yield of 1.5×10^7/ml, with a >98% neutrophil purity and a viability greater than 95%.

3.2. Respiratory burst assay

NBT reduction was not affected in any of the donors. There was statistical difference between PMA stimulated and non-stimulated neutrophils (****$p < 0.0001$), which is expected in neutrophils that have normal respiratory burst function (Fig. 1).

3.3. Neutrophil extracellular traps release

When evaluating NETs formation, neutrophils that were not stimulated did not form NETs (Fig. 2A and B), neutrophils exposed to PMA formed cloud-like structures that occupy 2-fold greater area compared to the initial cell size (Fig. 2C and D). Neutrophils exposed to *S. aureus* (Fig. 2E and F) formed elongated thin filaments that resembled extracellular traps. This structures were visualized with safranin under light microscopy and DAPI under a fluorescent invertoscope. When evaluating the counter stain of NBT-safranin neutrophils incubated for 30 min did not form NETs, there was only the formation of formazan crystals (Fig. 3A). Neutrophils incubated for 90 min had formation of formazan crystals and the formation of elongated thin filaments that were been released from the neutrophils along with nuclear condensation (pyknosis)(Fig. 3B) which are characteristics of NETosis [18].

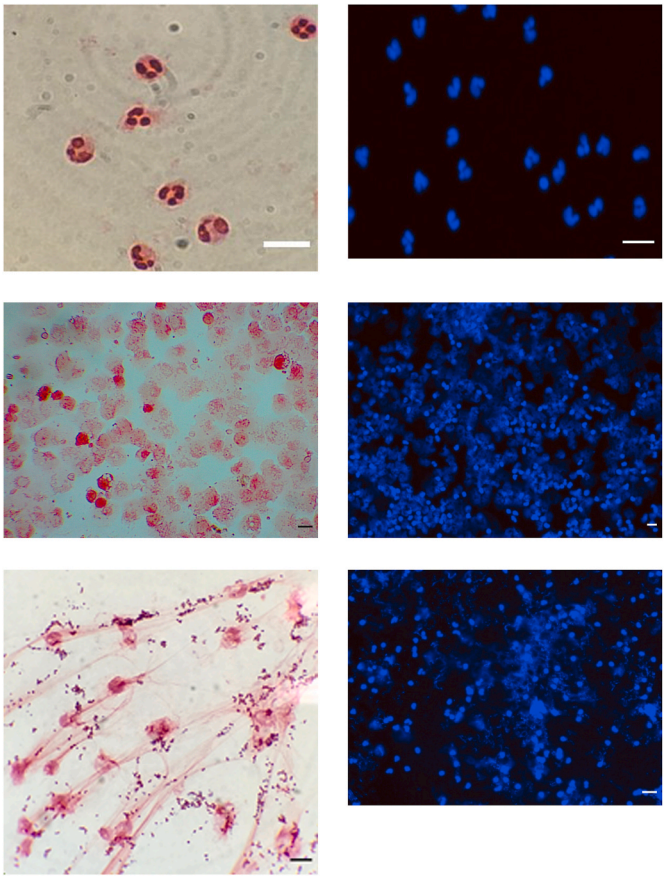

Fig. 2. NETs formation visualized with safranin. NETs were visualized under light microscopy using safranin 0.5% stain and fluorescence microscopy using DAPI. (A,B) Neutrophils stained with safranin 0.5% and DAPI after 3 h of incubation with no stimulus added (negative control). Magnification: 100x. (C,D) Neutrophils stained with safranin 0.5% and DAPI after 3 h of incubation with PMA (100 nM) (positive control). Magnification: 100x and 40x. (E,F) Neutrophils stained with safranin 0.5% and DAPI after 3 h of incubation with *S. aureus* (1,MOI:1:10) which was stained with crystal violet. Magnification: 100x and 40x. Scale bars = 10 μm. Representative images of experiments performed in triplicate. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

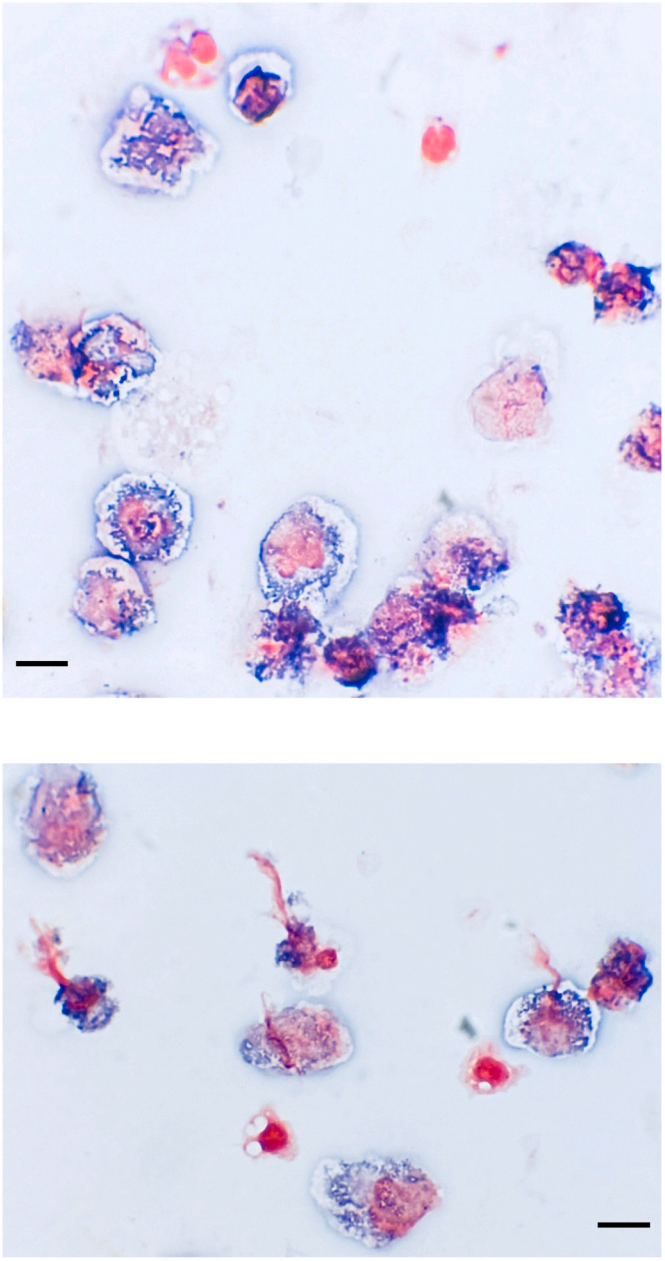

Fig. 3. NBT-safranin counter stain to visualize NETs. Neutrophils were incubated at 37 °C for 30 and 90 min with PMA (100 nM) and NBT 0.1%. (A) Neutrophils incubated for 30 min did not form NETs, there was only the formation of formazan crystals. (B) Neutrophils incubated for 90 min had formation of formazan crystals and the formation of elongated thin filaments. Magnification: 100x. Scale bars = 10 μm. Representative images of experiments performed in triplicate.

4. Discussion

4.1. Respiratory burst assay

NBT reduction is an excellent way to test neutrophil function, we based our method in one previously described [17]. By analyzing NBT reduction in a spectrophotometer we can obtain better information than using the traditional method, which is operator dependent. Since we observed no difference in the NBT reduction between the donors we expected to observe a normal NETs formation.

4.2. Neutrophil extracellular traps release

Safranin was used in a previous study that focused on simple staining of cells on a chip [19], we decided to use this stain to visualize NETs and compare it with DAPI. To our understanding this is first time that safranin is used to stain and visualize NETs. Safranin has a preference for the guanine-cytosine clusters [13] compared to DAPI that has a preference to adenine-thymine clusters [20]. Despite this differences, both of them gave very similar results in the visualization of NETs, hence NETs can be studied using safranin under light microscopy before employing more expensive procedures, making NETs research more cost effective. Previous studies have demonstrated the staining of NETs using Giemsa [21,22] and we wanted to test if using another simple stain like safranin could obtain similar if not better results. This procedure enhanced the sharpness of the images and extended the shelf life of these slides for more than 2 weeks by preventing rapid fading. With the implementation of our NBT-safranin stain we were able to confirm that safranin was indeed staining DNA because we observed how the DNA fiber was emerging from the neutrophil. However, this staining procedure by itself cannot confirm that the fibers you observe are NETs, hence other methods must be used to validate the presence of NET-components like myeloperoxidase, elastase or citrullinated histones [1,9,23–26]. This experiments are being developed by our lab to have a staining method equal to the standard methods. However, this method is suitable for preliminary experiments.

5. Conclusion

Our method demonstrates another way to visualize NETs that can be added to the standard methods of visualization of NETs, increasing the opportunities to generate knowledge in the topic in any lab around the world.

Ethics approval and consent to participate

All procedures were reviewed and approved by the Research and Ethics Committee of our institution with the number IN18-0005. All participants provided a written informed consent.

Authors' contributions

The authors confirm contribution to the paper as follows: study conception and design: Antonio Muñiz-Buenrostro, Alma Y. Arce-Mendoza; data collection: Edgar I. Montes-Zapata, Rubi C. Calderón-Meléndez, Hector A. Vaquera-Alfaro, Junior A. Huerta-Polina, Maricruz J. Montelongo-Rodríguez; analysis and interpretation of results: Antonio Muñiz-Buenrostro, Edgar I. Montes-Zapata, Rubi C. Calderón-Meléndez, Hector A. Vaquera-Alfaro, Junior A. Huerta-Polina, Maricruz J. Montelongo-Rodríguez; draft manuscript preparation: Antonio Muñiz-Buenrostro[1], Rubi C. Calderón-Meléndez, Hector A. Vaquera-Alfaro, Junior A. Huerta-Polina. All authors reviewed the results and approved the final version of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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