Video Article

A Simple Fluorescence Assay for Quantification of Canine Neutrophil Extracellular Trap Release

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Abstract

Neutrophil extracellular traps are networks of DNA, histones and neutrophil proteins released in response to infectious and inflammatory stimuli. Although a component of the innate immune response, NETs are implicated in a range of disease processes including autoimmunity and thrombosis. This protocol describes a simple method for canine neutrophil isolation and quantification of NETs using a microplate fluorescence assay. Blood is collected using conventional venipuncture techniques. Neutrophils are isolated using dextran sedimentation and a density gradient using conditions optimized for dog blood. After allowing time for attachment to the wells of a 96 well plate, neutrophils are treated with NET-inducing agonists such as phorbol-12-myristate-13-acetate or platelet activating factor. DNA release is measured by the fluorescence of a cell-impermeable nucleic acid dye. This assay is a simple, inexpensive method for quantifying NET release, but NET formation rather than other causes of cell death must be confirmed with alternative methods.

Video Link

The video component of this article can be found at <http://www.jove.com/video/54726/>

Introduction

There are over 70 million pet dogs in the USA alone¹. As valued family members, these animals often receive cutting edge medical care. Equally because they share our environment, dogs can provide insights into the pathogenesis and treatment of human disease¹. However, whether translating discoveries in human medicine into veterinary treatments or vice versa, it is important to thoroughly characterize species variations even in highly conserved systems such as the innate immune response. Examples of differences between the canine and human innate immune system include high expression CD4 on dog neutrophils²; the absence of a functional homolog of the cytoplasmic flagellin sensor IPAF in dogs³ and the expression of a caspase 1/4 hybrid in carnivores⁴.

Neutrophil extracellular traps (NETs) are a relatively recently discovered component of innate immunity⁵. NETs are networks of DNA, nuclear and granular proteins released in response to a wide range of inflammatory or infectious stimuli⁶. NET-like structures have been demonstrated across many species including chickens⁷, fish⁸, mollusks⁹ and acoelomates¹⁰, but there are species variations. For example, murine neutrophils respond more slowly to NETosis stimuli than human neutrophils, and form less diffuse NETs¹¹. There is a large body of evidence from multiple species that NETs entrap microbes, and more controversially may be directly involved in killing pathogens^{12,13}. However, NET components also enhance tissue damage, promote thrombosis and act as autoantigens^{14,15}. The balance between the beneficial and deleterious effects of NETs may vary between different diseases and different species, suggesting it is important to investigate NETs both in the species and condition of interest.

Here we describe a simple protocol for inducing and measuring the release of NETs by canine neutrophils. This method is similar to those used to isolate neutrophils¹⁶ and induce NETosis in other species, but conditions such as agonist concentration and incubation time have been optimized for canine neutrophils. A similar NET quantification DNA release assay has also been described in other species but the method presented here is also optimized for dogs^{8,17,18}.

Protocol

All experiments were performed with ethical permission from the Iowa State University Institutional Animal Care and Use Committee.

1. Blood Collection

1. Draw 9 ml of blood from a saphenous, cephalic or jugular vein directly into anticoagulant (e.g., ethylenediaminetetraacetic acid (EDTA), 1.8 mg K₂EDTA/ml blood) vacutainers, or into a syringe with immediate transfer to EDTA-containing blood tubes. Gently roll or invert the blood tubes to ensure adequate mixing of the blood and anti-coagulant.

2. Neutrophil Isolation

1. In a 50 ml conical tube, mix blood with an equal volume of sterile room temperature 3% dextran-500 (made up in 0.9% sterile saline) by gentle inversion. Leave standing upright for 18-20 min at room temperature. Transfer the straw colored upper layer to a fresh tube until it can no longer be collected without contamination by the lower red layer. The erythrocytes in the original tube can be discarded.
2. Centrifuge the supernatant at 500 x g for 10 min at 4 °C. Draw off and discard the supernatant. Manually re-suspend the cell pellet in 10 ml room temperature sterile phosphate buffered saline (PBS). Note that vortexing should not be used to re-suspend neutrophils at any stage of this protocol. Manually drawing off, rather than pouring off supernatants is also recommended throughout the protocol to maximize cell yield.
3. Add 10 ml of a room temperature polysucrose and sodium diatrizoate cell separation solution (e.g., Histopaque 1077) to a 50 ml conical tube. Carefully layer the cell-containing solution over the cell separation solution. Note that using cell separation solution which has not equilibrated to room temperature may reduce cell yield.
4. Centrifuge at 300 x g for 30 min at room temperature with the brake off.
5. As neutrophils are in the lowest layer of the gradient, draw off and discard the upper 3 layers, composed of an upper layer of plasma and platelets, a narrow white band of mononuclear cells and a clear layer of the density gradient medium.
6. To lyse any remaining erythrocytes, re-suspend the neutrophil pellet in 10 ml of room temperature water.
7. After 30 sec, restore tonicity by adding 10 ml of sterile room temperature 1.8% sodium chloride and mix by gentle inversion.
8. Centrifuge at 500 x g for 5 min at 4 °C. Draw off the supernatant and discard.
9. If the pellet is still red, repeat the lysis step. If the pellet is white (or if lysis has already been performed twice), gently re-suspend cells in 10 ml of sterile PBS.
10. Count cells using an automated counter or a hemocytometer. Typical yields are at least 10⁶ cells per ml of blood.
11. Centrifuge at 500 x g for 5 min at 4 °C. Draw off the supernatant and discard.
12. Re-suspend cells to the desired concentration in sterile room temperature PBS. For the DNA release assay described in section 2, resuspend at 5 x 10⁶ cells per ml.
13. If desired, transfer a small volume of cells directly or using a cytocentrifuge to a glass slide. Allow cells to dry and stain using a rapid fixation and staining kit according to the manufactures' instructions. If the neutrophil isolation has been successful, when examined under the microscope at least 95% of nucleated cells should be granulocytes (neutrophils, basophils, eosinophils) with minimal erythrocyte contamination.

3. DNA Release Assay

1. Set up the DNA release assay immediately after neutrophil isolation.
2. If cell clumping is present on examination of the stock solution by light microscopy, pass the cell suspension through a sterile 70 µm sterile filter immediately before setting up the assay.
3. To each test well of a sterile 96 well plate, add 5 x 10⁴ cells/well; agonist and Roswell Park Memorial Institute-1640 (RPMI) medium without phenol red and supplemented with 0.5% heat-inactivated fetal calf serum to a final volume of 100-200 µl. Include at least two blank wells per agonist in which the neutrophil stock solution is replaced by an equal volume of PBS. Note that setting up blank wells for each agonist is important in ruling out DNA contamination of the agonist or interference by the agonist with the fluorescence assay.
4. Incubate at 39 °C for 30 min in a carbon dioxide (4%) incubator.
5. Add agonists at desired concentrations and a cell impermeable nucleic acid dye. Note the optimal concentration of cell impermeable dye will vary between different nucleic acid dyes. Non-stimulated controls in which agonists are replaced by an equal volume of media should be included in each experiment.
NOTE: It is desirable to dilute agonists in similar volumes of culture medium to maintain consistent conditions between wells. Final well volumes of 200 µl have been used successfully and if this is altered, well volumes should remain identical for all conditions. Phorbol-12-myristate-13-acetate (PMA) (final concentration ≥0.1 µM) or platelet activating factor (PAF) (final concentration ≥31 µM) can be used as positive controls. Agonists should be measured at least in duplicate.
6. Incubate at 39 °C. Measure fluorescence using a microplate reader after 2 hours or at desired intervals. Note that optimal time intervals will vary with agonists used.
NOTE: Wavelength will depend on dye employed.
7. Subtract blank fluorescence before calculating mean fluorescence.
8. To allow comparison between individuals and between plates, calculate a fold change in fluorescence by dividing the mean fluorescence of stimulated wells by the mean fluorescence of non-stimulated wells.

Representative Results

Using this protocol, there should be a strong fold change in fluorescence after stimulating neutrophils with the positive controls, PMA and PAF. As illustrated in **Figure 1B**, stimulation of canine neutrophils with 31 µM PAF for 1 hr results in a mean 4.0-fold increase in fluorescence compared with non-stimulated cells (range 2.0-5.8, n = 5 dogs)¹⁸. PMA at 0.1 µM (**Figure 1A**) is a slower agonist which does not result in an increase in fluorescence until 2 hr but ultimately produces a similar fold increase in fluorescence (mean: 3.3, range 1.36-5.4, n = 5 dogs)¹⁸. Despite its slower kinetics, there are advantages to PMA as a positive control: it is relatively inexpensive compared with high concentrations of PAF and it has been widely used in murine and human studies.

Fluorescence of nucleic acid dyes is not specific for NET formation as it will also occur if there is cell death by other mechanisms or if reagents are contaminated with DNA. As shown in **Figure 2**, a commercial LPS preparation produced very high fluorescence in the absence of neutrophils, presumably due to contamination with bacterial DNA. Confirming NET formation through other methods such as immunofluorescence is recommended.

Figure 1: PMA and PAF increase fluorescence of a cell impermeable nucleic acid dye with different kinetics. Neutrophils were stimulated with 0.1 μ M PMA (**A**) or 31 μ M PAF (**B**) in the presence of the cell impermeable nucleic acid dye. Fluorescence was measured at hourly intervals and the fold change in fluorescence of stimulated cells compared to non-stimulated cells calculated. Fluorescence increased significantly between 1 and 2 hr for PMA (repeated measures one-way ANOVA followed by multiple t-tests with Bonferroni's correction, multiplicity adjusted $p = 0.04$; error bars are mean $\pm$ standard error for 5 individuals) but had already plateaued for PAF by 1 hr. This figure has been modified from Jeffery U, *et al.* Dogs cast NETs too: Canine neutrophil extracellular traps in health and immune-mediated hemolytic anemia. *Veterinary Immunology and Immunopathology*, 168 (3-4), 262-8, doi: 10.1016/j.vetimm.2015.10.014 (2015)¹⁸. [Please click here to view a larger version of this figure.](#)

Figure 2: Blank wells are important to rule out DNA contamination or interference by the agonist. A commercial source of LPS was added to acellular wells containing RPMI. A large fold change in fluorescence compared with wells containing non-stimulated neutrophils suggests contamination of the agonist with bacterial DNA. [Please click here to view a larger version of this figure.](#)

Discussion

The DNA release assay presented is a readily quantifiable assay for extracellular DNA. The method was adapted from similar techniques used to assess NET formation in other species, but centrifugation speeds, agonist concentrations and incubation times have been altered to optimize the method for use with canine neutrophils^{8,17,18}. Similar adjustments could be made to adapt the method for other species. The assay is straightforward and inexpensive when compared with other methods for measuring NETosis such as flow cytometry or image analysis. The method also does not require antibodies against NET components, so is ideal for use in dogs for whom commercially produced, validated species-reactive antibodies are often unavailable.

The major limitation of the method is the lack of specificity of cell-impermeable nucleic acid dyes for NET formation. These dyes also enter dead and dying cells, bind DNA and fluoresce. Therefore, fluorescence is not specific for NET formation. Use of other techniques (e.g., immunofluorescence) in parallel with the quantitative assay to visually confirm agonists are inducing NET formation rather than other forms of cell death is strongly recommended. The assay can also potentially be used as a general screening assay for cell death, for example as a means of quantifying cytotoxicity *ex vivo*.

The protocol is simple, but there are critical steps necessary to ensure success. Firstly, as noted in the protocol, to avoid excessive cell loss during neutrophil isolation it is important that supernatants are drawn off with a pipette rather than discarded by inverting tubes. Secondly, success of the assay relies on the non-stimulated control cells remaining in a viable and resting state throughout the incubation. Neutrophils can become activated at any stage during the protocol so careful attention should be paid to atraumatic blood collection technique and avoiding traumatic handling (e.g., vortexing) during neutrophil isolation. It is also important to remember that neutrophils have a limited lifespan so delay in isolating cells or setting up the assay should be avoided¹⁹. If prolonged incubation with agonists is attempted, viability of the non-stimulated cells over this time period should be confirmed.

As shown in **Figure 1**, there is considerable variation between dogs in their response to NET agonists. However, the assay should be reasonably consistent between replicates from the same animal, with an intra-assay variation of 9% based on 10 duplicates¹⁸. If there are large variations between replicates, cell clumping is likely responsible. Measures to reduce cell clumping include maintaining cells in PBS rather than a calcium

containing buffer prior to setting up the DNA assay, treating cells gently throughout isolation, and filtering the cell stock suspension prior to plating cells.

If these steps are followed, this protocol allows quantification of NET formation in response to a wide range of experimental conditions and agonists. Such studies can deepen our understanding of the role of NETs in canine health and disease. For example, this technique allows comparison of NET release between healthy dogs and those with immunosuppressive or autoimmune diseases in which NETosis may be impaired or enhanced. Alternatively, when combined with other techniques such as immunofluorescence, the assay can be used to identify novel agonists capable of inducing NETosis and novel inhibitors of NETosis.

Disclosures

The authors have nothing to disclose.

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