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Sandwich ELISA for Circulating Myeloperoxidase- and Neutrophil Elastase-DNA Complexes Released from Neutrophil Extracellular Traps

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Abstract

Neutrophil extracellular traps (NETs) are DNA scaffolds released by activated neutrophils that contain enzymes from neutrophil granules, such as myeloperoxidase (MPO), neutrophil elastase (NE) and cathepsin-G. NETs are produced by neutrophils in response to various stimuli, including invasion of pathogenic microorganisms. Here we describe a new ELISA method for quantifying the circulating levels of MPO- and NE-associated DNA in human plasma.

Keywords: ELISA; NETs; Myeloperoxidase; Neutrophil elastase; DNA

Introduction

In 2004, Brinkmann et al. discovered a novel cell death program that involved activated neutrophils releasing web-like structures composed of decondensed chromatin associated with histones and neutrophil granule proteins, such as neutrophil elastase (NE), cathepsin G, myeloperoxidase (MPO) and lactoferrin [1]. These web-like structures are known as neutrophil extracellular traps (NETs) and the process of NET extrusion by neutrophils is called NETosis [1,2]. Release of NETs is mediated by various stimuli, which include microbial breakdown products, proinflammatory cytokines (interleukin (IL)-1, IL-8 and tumor necrosis factor-α) and activated platelets [3,4].

NETs provide a high local concentration of antimicrobial molecules and these structures can trap, immobilize, and kill a wide range of invading microorganisms, including gram-negative and gram-positive bacteria [1], fungi [5], viruses [6] and protozoa [7]. Therefore, NETs have an important role in innate immunity, and it has been shown that deficiency of NET production or destruction of the NET skeleton by DNase increases the risk of infection [8].

Although interest in NETs has increased recently, there is no reliable assay for detecting NETs in the clinical setting. We developed a modified sandwich enzyme-linked immunosorbent assay (ELISA) to quantitatively measure NETs in human plasma based on the method of Kessenbrock et al. [9]. Using specific antibodies for MPO or NE as the capture antibodies and a DNA-specific detection antibody, this assay can identify circulating MPO-DNA and NE-DNA complexes. Limited digestion of DNA in the samples is performed to increase binding of the capture antibody with circulating MPO- or NE-associated DNA. Detection of circulating MPO- or NE-associated DNA is more specific for NETs than circulating cell-free DNA, and the limited DNA digestion increases the sensitivity of this assay.

Materials and Method

This assay is based on quantitative detection of MPO- or NE-associated DNA by performing a "sandwich" ELISA with anti-MPO, anti-NE and anti-DNA monoclonal antibodies. A monoclonal antibody specific for MPO or NE is used to coat the wells of microtiter strips to capture MPO-associated DNA or NE-associated DNA derived from NETs. This study was conducted in conformity with the declaration of Helsinki and was approved by the Institutional Review Boards of Fujita Health University (#150). After receiving review board approval, written informed consent was obtained from each subject.

Materials and Preparation

Reagent

Coating Buffer

- M Carbonate-bicarbonate buffer, pH 9.6.
- Blocking Buffer
- 1% bovine serum albumin with 0.05% sodium azide in PBS.
- Other reagents:
- 0.5% Triton X
- 0.5 M EDTA

Antibody and immunoconjugate

- Anti-MPO antibody (Merck Millipore Corp., catalog # 07-496)
- Anti-NE antibody (Merck Millipore Corp., catalog # MABF759)
- Peroxidase-conjugated anti-DNA antibody (Roche Diagnostics, Indianapolis, IN, USA; Cell death Detection ELISA #1154467500: bottle 2)
- ABTS/buffer solution (100 mg ABTS in 100 ml of 3.25 mM sodium perborate, 39.8 mM citric acid, and 60 mM disodium hydrogen phosphate (pH 4.4-4.5), Roche Diagnostics, # 10102946001)
- DNase I (New England BioLabs, #M0303L)

Other materials

- 96-well microtiter plate (Nunc™ 467466 Immuno™)

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- Adhesive plastic cover
- Microtiter plate reader capable of reading wavelengths from 405-490 nm.
- Absorbent paper towel
- Shaker

Preparation of reagents

Coating buffer: Add 10.6 g of sodium carbonate and 8.4 g of sodium bicarbonate to approx. 900 ml of distilled water in a beaker. Measure the pH of the solution using a pH meter and adjust it to 9.6 by adding a weak acid or alkali as required. Then add further distilled water to make up a total volume of 1000 ml. Store the coating buffer in the refrigerator at 4°C.

Blocking buffer: Add approx. 70 ml of PBS and 250 µl of 20% sodium azide to 1 g of BSA, mix gently, and then adjust the total volume to 100 ml. Let the solution to stand for 10 min after mixing.

Washing solution (0.5% Triton X): The viscosity of 100% Triton X is very high. It is better to use a plastic pipette and cut the tip to make it wider if possible. Allow the 10% solution to stand for 1 day before use. Sterilization and addition of a preservative are not required.

Peroxidase-conjugated anti-DNA antibody: Reconstitute the lyophilizate in 1 ml of double distilled water and mix thoroughly for 10 min. Store it at -80°C.

Sample collection and storage

Collect blood from septic shock patients and healthy volunteers into a tube containing an anticoagulant such as EDTA, heparin, or ACD and centrifuge at 3000 rpm for 5 min at 4°C. Store the plasma thus obtained at -80°C until further use.

Assay Method

First day

- Dilute the anti-MPO antibody or anti-NE antibody at 1:2000 in coating buffer (pH 9.6).
- Add 100 µl of diluted antibody to each well of the plate. Several points should be considered when coating an ELISA plate. The coating buffer should not contain any kind of detergent to ensure equal and smooth binding of antibody to the walls of each well. In addition, an excessively high concentration of coating protein occasionally reduces binding and this phenomenon is known as the "hook" effect. The typical concentration range of protein coating solutions is 2-10 µg/ml.
- Cover the plate with an adhesive plastic cover and incubate overnight at 4°C.

Second day

- Thoroughly remove the coating solution from the wells, and wash the plate three times by filling each well with 300 µl of PBS. Then excess PBS is removed by patting the plate dry with a paper towel.
- Pipette 200 µl of blocking buffer into each well and cover the plate tightly with an adhesive plastic cover.
- Incubate for 90 to 120 min at room temperature to block the wells.
- Thoroughly remove the solution from the wells, and wash the plate three times by filling each well with 300 µl of PBS. Then excess PBS is removed by patting the plate dry with a paper towel.
- Add 25 µl of plasma to each well. Then add 75 µl of PBS to each well for 4-fold dilution of the plasma. Thaw samples and bring them to room temperature before measurement, and avoid repeated thawing and freezing of samples.
- Shake the plate once on a shaker adequately to mix the samples in the wells.
- Add 0 to 3 µl of 100-fold diluted DNase (0.3 mg/ml) to each well. The final concentration of DNase is 0 to 0.9 µg/ml reaction mixture. Cover the plate with an adhesive plastic cover and shake it on the shaker to thoroughly mix the samples in the wells.
- Incubate for 15 min at room temperature.
- Remove the plastic cover and add 1 µl of 0.5 M EDTA to each well to stop the DNase reaction. Cover the plate with an adhesive plastic cover and shake it adequately for 15 s. Then incubate the plate overnight at 4°C.

Third day

Bring the peroxidase conjugated anti-DNA antibody and incubation buffer from the cell death detection ELISA kit to room temperature.

Dilute 10% Triton X by 20-fold with distilled water to 0.5%.

- Discard the plastic cover and remove the solution from the wells by suction or tapping.
- Rinse the wells 3 to 4 times with washing solution (300 µl per well) and remove the rinsing solution carefully and thoroughly.
- Dilute the peroxidase-conjugated anti-DNA antibody to 1:40 with the incubation buffer supplied.
- Add 100 µl of the diluted peroxidase-conjugated anti-DNA antibody to each well and cover the plate with an adhesive plastic cover.
- Incubate the plate for 90 min at room temperature.
- Remove the solution from the wells thoroughly by tapping or suction.
- Rinse the wells three times with the washing solution (300 µl per well) and carefully remove the residual solution.
- Add 100 µl of ABTS/buffer solution to each well and cover the plate with an adhesive plastic cover.
- Incubate the plate in the dark on a shaker at 250 rpm until sufficient color has developed for photometric analysis (about 20 min).
- Homogenize the contents of the wells by carefully tapping the side of the plate.
- Measure the OD of each well at a wavelength of 405 nm. A wavelength of 490 nm can be used for reference (optional).

Results and Discussion

During primary incubation with plasma samples containing

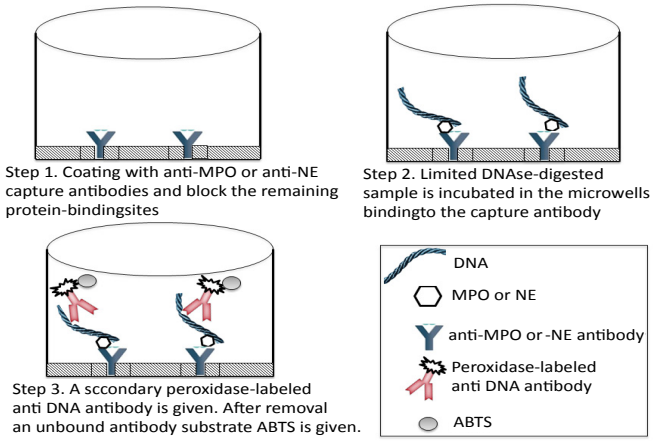
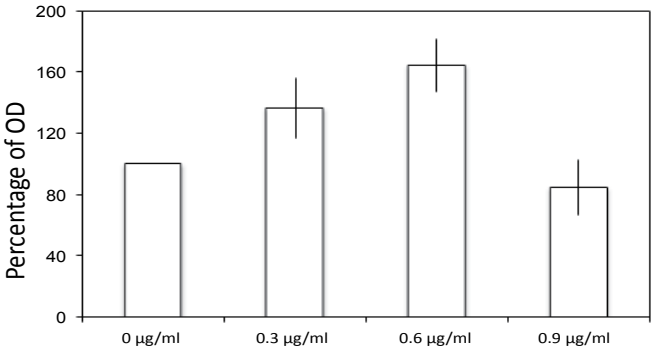


Figure 1: Sandwich ELISA for measuring MPO-DNA and NE-DNA complexes.



Data is calculated as percentage of the OD in the absence of DNase and expressed as mean ± SD

Figure 2: Limited digestion of DNA by DNase.

MPO-or NE-associated DNA, MPO or NE will bind to one site of the capture antibody. After washing, secondary incubation is done with a peroxidase conjugated anti-DNA monoclonal antibody to complete the "sandwich". After removal of excess secondary antibody, a peroxidase substrate (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid: ABTS) is added, which reacts with the bound peroxidase enzyme to yield a soluble green product that is detected at 405 nm (Figure 1).

In NETs, MPO and NE proteins are attached to long threads of chromatin. Limited digestion of DNA with the enzyme DNase cuts the chromatin threads into shorter pieces and increases binding between the capture antibody and MPO or NE associated with DNA. A high DNase concentration or digestion for too long could result in excessive digestion of DNA and thus reduce the absorbance. To assess the optimum conditions for DNA digestion, samples containing MPO- and NE-associated DNA were incubated with increasing concentrations of DNase and the reaction was stopped after 15 min by adding EDTA. The highest optical density (OD) was observed when DNase was added at 0.6 µg/ml (Figure 2). Therefore, limited DNA digestion was done with 0.6 µg/ml reaction mixture of DNase for 15 min at room temperature.

The plasma concentrations of MPO- and NE-DNA from seven septic shock patients showed 11.2 ± 1.8 and 9.9 ± 4.3 absorbance/ml, respectively. While plasma concentration of MPO- and NE-DNA from four healthy volunteers showed 1.15 ± 0.91 and 1.4 ± 1.5 absorbance/ml, respectively. Data were expressed as mean ± SD.

Conclusion

This new ELISA assay is based on quantitative detection of MPO- or NE-associated DNA by performing a "sandwich" ELISA with anti-MPO, anti-NE, and anti-DNA monoclonal antibodies. A monoclonal antibody specific for MPO or NE is used to coat the wells of microtiter strips to capture MPO-associated DNA or NE-associated DNA derived from NETs. This sandwich ELISA assay is reliable and useful method for investigating the characteristics of NETs in human samples.

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