Quantification of Neutrophil Extracellular Trap Formation Using an ELISA Specific for Citrullinated Histone H3

J.L. Mobley, J.M. Rumble, P.J. Domanski, J.S. Bickel, and M.C. Doty

Cayman Chemical, Ann Arbor, MI, USA

Abstract

The objective of this study was to develop an ELISA specific for citrullinated histone H3 (CitH3) that could be used to accurately and reproducibly quantify NETs from multiple species.

The detection and relative quantification of NET formation has relied primarily upon visualization by fluorescence microscopy, which is not accurately quantifiable, or measurement of extracellular DNA, which is quantifiable, but not exclusive to NET formation. Attempts have been made to quantify NET formation by using heterobifunctional ELISAs detecting various combinations of myeloperoxidase, neutrophil elastase, and DNA, but these suffer from a lack of reliable, reproducible standards. In addition, these components cannot serve as exclusive biomarkers for NET formation, since they are also released from neutrophil granules or during necrotic cell death. CitH3, however, can serve as a NET-specific biomarker, since it is solely produced during NET formation. Histone H3 undergoes citrullination catalyzed by enzyme peptidylarginine deiminases (PAD4 and PAD2). Hyper activity of PADs leads to the development of NETs. Therefore, CitH3 quantification by ELISA provides a quantitative, reproducible, selective, and inexpensive method for assessing NET formation.

A monoclonal antibody (11D3) was produced against the tail of histone H3 citrullinated at arginines 2, 8, and 17). A second monoclonal antibody (2D6) that is specific for a highly-conserved epitope on unmodified histone H3 was isolated from a mouse (NZB/W F1) with spontaneous lupus. These antibodies form a sandwich pair that recognizes CitH3 that is released during the process of NET formation. PAD4-citrullinated HeLa core histone H3 is used as a quantifiable standard in the ELISA.

This Citrullinated Histone H3 ELISA can detect and quantify CitH3 from NETs produced by human peripheral blood neutrophils and mouse bone marrow neutrophils after treatment of the NETs with S7 nuclease to release the DNA-bound CitH3. The ELISA also detects CitH3 from the plasma of LPS challenged mice. CitH3 can be detected in the detergent-insoluble fraction of neutrophil lysates early after neutrophil stimulation, but prior to mature NET formation.

Introduction

- Neutrophil extracellular traps (NETs) are web-like structures extruded by neutrophils in response to infectious challenge that are composed of DNA, histones, and multiple anti-microbial agents (MPO, elastase, defensins, etc.).
- Most NET components are also released in soluble form from neutrophil granules (MPO, elastase, defensins) or during necrotic cell death (DNA and histones). Thus, these analytes are not NET-specific, and their detection from biofluids cannot be used accurately as a surrogate measurement of NET formation.
- Citrullinated histone H3 (CitH3) is one NET component that is produced almost exclusively during NET formation, and thus, could be considered a NET-specific biomarker.
- We have developed an ELISA that can be used to measure CitH3 as a means of quantifying NET formation.
- This ELISA is a sandwich assay that employs a capture monoclonal antibody directed against the citrullinated tail of histone H3 and a detection monoclonal antibody directed against a conserved epitope on the body of histone H3 (**Figure 1**).

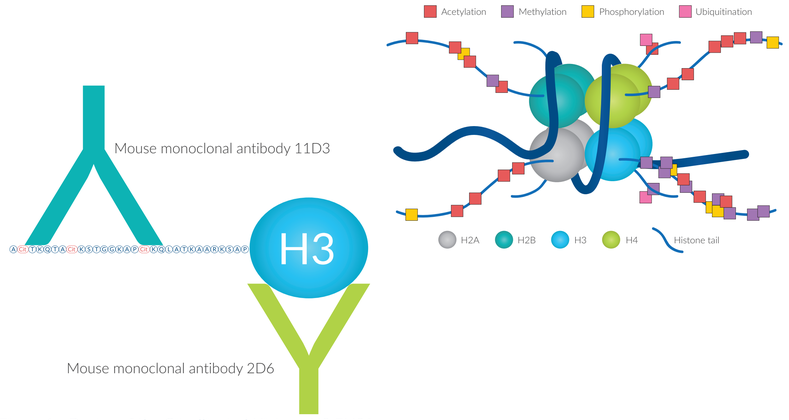


Figure 1 - Design of the Citrullinated Histone H3 ELISA.

Experimental Methods

Generation of a monoclonal antibody specific for citrullinated histone H3

BALB/c mice were immunized with the 30 amino-acid tail of histone H3 with citrulline replacing arginine at positions 2, 8, and 17. Hybridomas were screened for their ability to recognize the tri-citrullinated tail peptide, but not the unmodified, native tail or any combination of mono- or di-citrullinations. One monoclonal antibody, 11D3, met these qualifications (**Figure 2**). This antibody also detects citrullinated histone H3, but not unmodified H3, by Western blot. It is available from Cayman as Histone H3 (Citrullinated R2 = R8 + R17) Monoclonal Antibody (Cayman Item No. 17939).

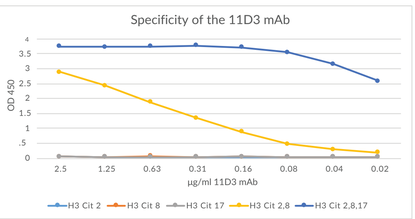


Figure 2 – Specificity of the 11D3 monoclonal antibody.
Various modifications of the histone H3 tail peptide were coated onto Nunc MaxiSorp™ wells and tested for binding of the 11D3 mAb, using a goat-anti-mouse IgG-HRP detection antibody. 11D3 did not bind at all to unmodified peptide, or to the 30-mer containing a single citrulline at position 2, or 8, or 17. The peptide citrullinated at position 2 and 8 (but not 17) had weak binding to 11D3, approximately 100-fold less than binding to the tri-citrullinated peptide at 2, 8, and 17.

Generation of a monoclonal antibody specific for native histone H3

Histones are highly conserved between species, making it difficult to produce high-quality anti-histone antibodies by immunizing one species with histones from another, especially if the histones are not denatured. Thus, we chose to use lupus-prone NZB/W F1 mice. These mice spontaneously produce anti-dsDNA and anti-histone antibodies, resulting in a lupus-like pathology that eventually kills the mice.

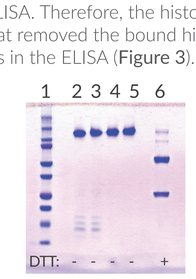
At the first indication of disease (lethargy, ruffled fur) the spleen of an NZB/W F1 mouse was harvested and fused with the X63-Ag8.653 fusion partner. The resulting hybridoma supernatants were initially screened for binding to human nucleosomes. The 63 positive wells were further screened for binding to specific recombinant histones (**Table 1**). Two of the H3-specific hybridomas, 2D6 and 7G10, were found to recognize H3, either citrullinated or unmodified, from multiple species.

Table 1 - Screen for mAbs specific for individual histones

ELISA screening reagent	Vendor	# Positive	% Positive
Primary screen			
Core Histones (human)	Cayman Item No. 11010	63 of 600	10.5
Secondary screen			
Histone H2A (*Xenopus* recombinant)	Cayman Item No. 10261	5 of 63	7.9
Histone H2B (*Xenopus* recombinant)	Cayman Item No. 10262	7 of 63	11.1
Histone H3 (human recombinant)	Cayman Item No. 10263	27 of 63	42.8
Histone H4 (human recombinant)	Cayman Item No. 10264	10 of 63	15.9
Citrullinated H3 peptide 1-21, Cit 2/8/17	Anaspec	4 of 63	6.3
All 4 histones (pan histone)	See above	1 of 63	1.6
3 of 4 histones	See above	7 of 63	11.1
Calf thymus DNA	Sigma	13 of 63	20.6

Spleen cells from an unimmunized, 200-day old NZB/W F1 mouse were fused with the P3X63Ag8 myeloma cell line and plated in 600 wells. The supernatants were screened first for binding to HeLa nucleosomes. Strongly positive supernatants were screened again for binding to individual nucleosome components.

During the purification of both 2D6 and 7G10 from terminal hybridoma supernatants, it was discovered that histones released from the dying hybridoma cells were binding to the 2D6 and 7G10 antibodies. Having H3 already in the antigen-binding site of the antibodies prevents their purification by affinity chromatography, and prevents them from functioning as secondary antibodies in the ELISA. Therefore, the histone-contaminated mAbs had to be subjected to a purification step that removed the bound histone from the antibody before they could be used as detection antibodies in the ELISA (**Figure 3**).



1. MW standards
2. 3B11 mAb/Ag complex
3. 3B11 mAb/Ag complex
4. Purified mAb 3B11
5. Purified mAb 3B11
6. Purified mAb 3B11

Figure 3 – Anti-histone antibodies bind histones that are released from dying antibody-producing cells in terminal hybridoma supernatants.
A multi-histone reactive mAb (3B11) produced from NZBWF1 mice was purified by protein-A and analyzed by SDS-PAGE. The 3B11 antibody purified from terminal hybridoma supernatant carries with it multiple histone proteins (Lanes 2 and 3). These can be removed using a Cayman proprietary method (Lanes 4 and 5).

Identification of an ELISA standard

Recombinant human histone H3 (Cayman Item No. 10263) was citrullinated by recombinant human PAD4 (Cayman Item No. 10500) in a reaction buffer containing 0.1 M Tris, pH 7.4, 10 mM $CaCl_2$, and 5 mM DTT. The degree of citrullination was assessed using Cayman's Citrulline-specific Probe (Cayman Item No. 16172) and by mass spectroscopy. Core histones isolated from HeLa cells (Cayman Item No. 11010), containing two copies each of histones H2A, H2B, H3, and H4 were similarly citrullinated by recombinant human PAD4.

Development of the ELISA

The final version of the ELISA is sold as Cayman's Citrullinated Histone H3 (Clone 11D3) ELISA Kit (Cayman Item No. 501620). It uses Nunc MaxiSorp™ 96-well strip plates coated with 11D3 at 1 µg/well. Excess protein binding sites are blocked with a blocking buffer containing BSA and sucrose. The CitH3 standard is added in a range from 0.15 to 10 ng/ml. The 2D6 mAb is directly conjugated to HRP and used as a single-step detection. TMB is used as the HRP substrate.

Isolation of neutrophils

Human peripheral blood neutrophils were isolated using a combination of density centrifugation (Ficoll) and red blood cell lysis. Mouse neutrophils were isolated from peripheral blood, inflamed peritoneal exudate, or bone marrow using Cayman's Neutrophil (mouse) Isolation Kit (Cayman Item No. 601070.

NET generation and release of CitH3

Neutrophils were stimulated at 37°C for 1-5 hours in the presence of PMA (0-20 nM) or A23187 (0-25 µM) in RPMI-1640 containing 10% FCS and 1 mM $CaCl_2$ (activation medium). For release of CitH3 into the supernatant, S7 nuclease was added to the activation medium during the entire 4-hour stimulation period. At the end of the 4-hour incubation, the S7 nuclease was inactivated by the addition of EDTA.

For release of CitH3 from actively-forming NETs within cells, the stimulated neutrophils were pelleted by gentle centrifugation, and the cell pellets were lysed in M-PER lysis buffer (Sigma) containing protease inhibitors for 30 minutes on ice. The lysates were centrifuged at 12,000 x g to pellet the detergent insoluble material, including the CitH3-containing nucleosomes. The lysate was removed, and the insoluble fraction resuspended in culture medium containing S7 nuclease, and incubated at 37°C for 15-30 minutes. The S7 nuclease was inactivated by the addition of EDTA.

In some experiments, different activation medium formulations were tested including Cayman's NET Assay Buffer (RPMI-1640 with 0.5% BSA), a buffer formulation included in Cayman's NETosis Assay Kit (Cayman Item No. 601010).

Plasma from LPS-treated mice

BALB/c mice were injected with LPS intraperitoneally at 1 µg/mouse. The mice were anesthetized, and blood was collected from the inferior vena cava into heparin-containing syringes. The plasma was collected by centrifugation. Plasma was tested untreated, or treated prior to analysis with EDTA, S7 nuclease, or a combination of both.

Results

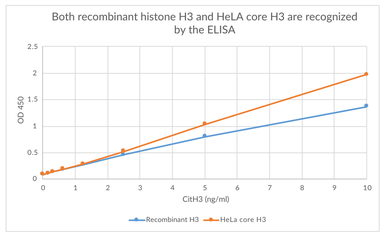
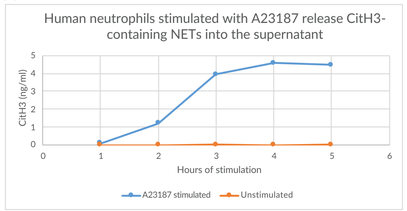


Figure 4 – Generation of the sandwich assay with two possible standards.
A sandwich ELISA was developed using the 11D3 mAb as the capture antibody and 2D6-HRP as the detection antibody. Either recombinant human histone H3 or HeLa core histones could function as an acceptable standard in the ELISA.

A



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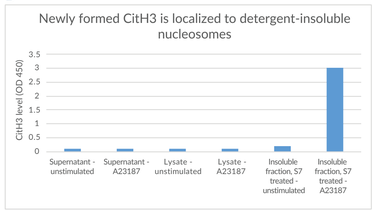

Figure 5 – Time-course of CitH3 production from human neutrophils.
(A) Human neutrophils stimulated with A23187 release CitH3 into the supernatant within 2 hours of stimulation, peaking at 4 hours. (B) Prior to 2 hours, CitH3 can be recovered from the detergent insoluble fraction of cell lysates, but not the supernatant.

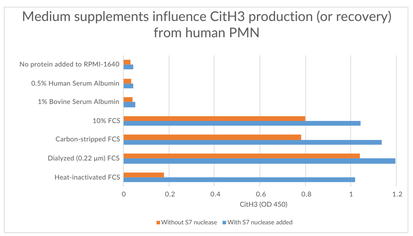

Figure 6 – Effects of BSA, HSA, or serum supplementation of RPMI on the generation of CitH3.
RPMI-1640 basal medium supplemented with 10% FCS supports CitH3 release from activated neutrophils in the presence of S7 nuclease and calcium. CitH3 cannot be detected if FCS is replaced with BSA or human albumin.

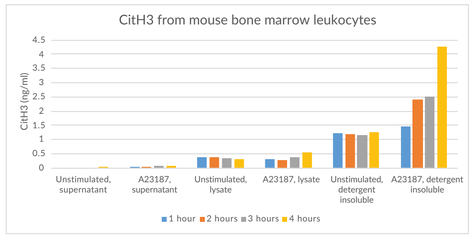

Figure 7 – Detection of CitH3 from mouse bone marrow leukocytes.
Unfractionated mouse bone marrow was stimulated with A23187 for 1-4 hours. CitH3 was detected in the insoluble fraction of detergent lysates and the cell supernatants.

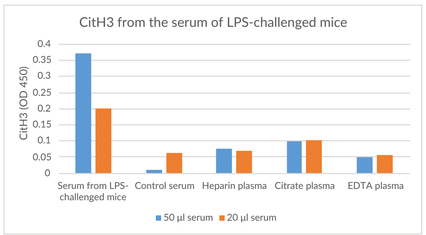

Figure 8 – Detection of CitH3 from the serum of LPS-challenged mice.
Serum of mice challenged with LPS was compared to control serum or control plasma with various anticoagulants for the presence of CitH3.

Conclusions

- An ELISA for CitH3 has been validated for use as a tool for quantifying NET formation.
- CitH3 can be detected in the nuclear fraction (detergent-insoluble) soon after neutrophil stimulation, but prior to NET release.
- CitH3 can be detected in the supernatants of stimulated neutrophils coincident with NET release.
- CitH3 can be detected in the serum of LPS-challenged mice, as a remnant of prior NET formation and disruption.