A Thermonuclease of *Neisseria gonorrhoeae* Enhances Bacterial Escape From Killing by Neutrophil Extracellular Traps

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Acute gonorrhea is characterized by neutrophilic inflammation that is insufficient to clear *Neisseria gonorrhoeae*. Activated neutrophils release extracellular traps (NETs), which are composed of chromatin and decorated with antimicrobial proteins. The *N. gonorrhoeae* NG0969 open reading frame contains a gene (*nuc*) that encodes a putatively secreted thermonuclease (Nuc) that contributes to biofilm remodeling. Here, we report that Nuc degrades NETs to help *N. gonorrhoeae* resist killing by neutrophils. Primary human neutrophils released NETs after exposure to *N. gonorrhoeae*, but NET integrity declined over time with Nuc-containing bacteria. Recombinant Nuc and conditioned medium from Nuc-containing *N. gonorrhoeae* degraded human neutrophil DNA and NETs. NETs were found to have antimicrobial activity against *N. gonorrhoeae*, and Nuc expression enhanced *N. gonorrhoeae* survival in the presence of neutrophils that released NETs. We propose that Nuc enables *N. gonorrhoeae* to escape trapping and killing by NETs during symptomatic infection, highlighting Nuc as a multifunctional virulence factor for *N. gonorrhoeae*.

**Keywords.** *Neisseria gonorrhoeae*; Nuc; thermonuclease; neutrophil; polymorphonuclear leukocyte; neutrophil extracellular traps.

The gram-negative diplococcus *Neisseria gonorrhoeae* causes the sexually transmitted infection gonorrhea. Worldwide, >100 million cases of gonorrhea are estimated to occur annually [1]. Gonorrhea can cause acute urethritis in males and cervicitis in females and lead to sequelae such as pelvic inflammatory syndrome, ectopic pregnancy, sterility, and, through vertical transmission, infant blindness [2]. There is no vaccine for gonorrhea, and antibiotic-resistant *N. gonorrhoeae* strains are emerging, raising global public health concern [3, 4].

One hallmark of *N. gonorrhoeae* infection is a potent neutrophil-driven inflammatory response [5]. Neutrophils are professional phagocytes that release antimicrobial species intracellularly or extracellularly to defend against internalized and extracellular pathogens, respectively [6, 7]. Neutrophils also release neutrophil extracellular traps (NETs), which comprise chromatin fibers associated with antimicrobial proteins [8]. NETs have been observed in vivo in models of acute inflammation and infection, and NETs capture and can kill bacteria and fungi [8, 9]. NETs may be released by dying cells by NETosis or from live, infected cells; mitochondrial DNA can also contribute to NETs [10–12]. *Neisseria gonorrhoeae* can be cultured from the disease exudates of patients, suggesting that a population of *N. gonorrhoeae* escapes neutrophil killing mechanisms [2]. We previously showed that some *N. gonorrhoeae* survive inside primary human neutrophils by residing in immature phagosomes [13]. We have also reported that most extracellular *N. gonorrhoeae* survive exposure to neutrophils [14], but it was unclear whether this was attributable to any specific mechanism.
Thermonucleases are heat-stable, calcium-dependent endonucleases implicated in the virulence of human pathogens, including *Staphylococcus aureus* [15, 16]. *Neisseria gonorrhoeae* encodes a thermonuclease homolog (Nuc) that degrades single- and double-stranded DNA and helps remodel *N. gonorrhoeae* biofilms [17]. Here, we show that *N. gonorrhoeae* induces NET formation from human neutrophils; however, Nuc degrades the DNA backbone of NETs over time. NETs contain antimicrobial proteins that are capable of killing *N. gonorrhoeae*, but the presence of Nuc enhances survival of *N. gonorrhoeae* after exposure to NET-producing neutrophils. These findings implicate Nuc as a virulence factor that protects *N. gonorrhoeae* from killing by neutrophils.

**MATERIALS AND METHODS**

**Bacterial Strains and Growth Conditions**
Piliated, opacity protein–deficient *N. gonorrhoeae* (strain FA1090) was used for most experiments [18]. An insertion-deletion mutation with a kanamycin resistance cassette was generated in *nuc* (NG00969), using plasmid pCTS#43 [17]. Transformants were selected on gonococcal medium base agar (GCB) containing Kellogg supplements I and II [19] with 50 µg/mL kanamycin and were confirmed by DNA sequencing. ∆*nuc* *N. gonorrhoeae* was complemented by transformation with pCTS#33 [17], and colonies were selected on supplemented GCB with 50 µg/mL spectinomycin. All *N. gonorrhoeae* isolates exhibited identical lipoooligosaccharide banding patterns on silver-stained polyacrylamide gels. *Neisseria gonorrhoeae* was cultivated on GCB at 37°C in 5% CO₂ overnight. Viable, exponential-phase *N. gonorrhoeae* was obtained by sequential dilution in rich liquid medium as described elsewhere [20]. Bacterial growth was monitored by diluting cultures to an OD₅₅₀ of approximately 0.07 and enumerating colony-forming units (CFU) per milliliter every hour for 3 hours. Parent, ∆*nuc*, and *nuc*-complement *N. gonorrhoeae* grew similarly in liquid medium (Supplementary Figure 1A). Piliated, Opa-expressing *N. gonorrhoeae* of strain 1291 and an isogenic ∆*nuc* mutant were also used [17]; immunoblotting revealed that the strains had identical Opa expression profiles (data not shown).

To collect conditioned medium, *N. gonorrhoeae* was grown in liquid medium as described above, except at the final dilution, cultures were inoculated into phenol red–free Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with l-glutamine (HyClone) at an OD₅₅₀ of 0.07 and grown to an OD₅₅₀ of 0.4. Bacterial suspensions were centrifuged at 10,000g for 3 minutes, and supernatants were passed through 0.2-µm filters.

**Isolation of Human Neutrophils**
Peripheral blood was obtained from healthy human donors. All provided informed consent, following an approved protocol by the University of Virginia Institutional Review Board for Health Science Research. Neutrophils were purified in endotoxin-free conditions using a Ficoll-Hypaque gradient and erythrocyte lysis as described elsewhere [21]. Neutrophils were suspended in ice-cold Dulbecco’s phosphate-buffered saline (without Ca²⁺ and Mg²⁺; Thermo Scientific) with 0.1% dextrose and used within 30 minutes of purification. Neutrophil preparations were >95% pure by phase-contrast microscopy. Replicate experiments used neutrophils from different donors.

**Neutrophil DNA Integrity Assay**
Neutrophil genomic DNA (3 µg) was incubated for 30 minutes at 37°C in molecular biology–grade water (Thermo Scientific) and DNase I reaction buffer alone, with 0.5 U of DNase I (recombinant bovine pancreatic DNase I; New England Biolabs), or with recombinant Nuc that was boiled for 10 minutes or left unboiled. Nuc was expressed in *Escherichia coli* and purified as described by Steichen et al [17]. In some experiments, Nuc was incubated for 30 minutes at 37°C with 0.1 mg/mL thymidine-3,5-bisphosphate disodium salt (Toronto Research Chemicals) prior to addition of neutrophil DNA. Samples were separated on a 1% agarose gel run at 4°C. DNA was visualized by ethidium bromide staining and imaged with ChemiDoc XRS+ (BioRad).

**NET Integrity Assay**
Neutrophils were suspended in RPMI 1640 medium (HyClone) and seeded (1 × 10⁶ cells/well) into 1.7-cm² wells of poly-l-lysine–coated chambered coverglasses (Nalge-Nunc International). To chemically induce NETs, neutrophils were treated with 20 nM phorbol 12-myristate 13-acetate (PMA; Sigma) for 1.5 hours.

**Treatment With *N. gonorrhoeae***
Untreated or PMA-treated neutrophils were exposed to parent, ∆*nuc*, or *nuc*-complement *N. gonorrhoeae* at a multiplicity of infection of 1 for 3 hours.

**Treatment With Nuc Protein**
Neutrophils that were exposed to PMA or ∆*nuc* *N. gonorrhoeae* for 1.5 hours were treated with indicated amounts of Nuc for 1.5 hours. Nuc was pretreated with thymidine-3,5-bisphosphate disodium salt (0.1 mg/mL) for 30 minutes, where indicated.

**Treatment With Conditioned Supernatant**
Neutrophils that were exposed to PMA or ∆*nuc* *N. gonorrhoeae* for 1.5 hours were treated with 200 µL of conditioned supernatant in RPMI 1640 medium (equivalent to approximately 2.5 × 10⁷ *N. gonorrhoeae* CFU/mL) for 1.5 hours. Supernatant was preincubated with thymidine-3,5-bisphosphate disodium salt as described above.
NET Visualization and Analysis

NETs were visualized using a protocol adapted from a previous study [22]. Neutrophils in chambered coverglasses (Nalge Nunc) were fixed in 4% paraformaldehyde and blocked overnight in 1% bovine serum albumin (Sigma) in phosphate-buffered saline at 4°C. Neutrophil elastase and LL-37 were detected using mouse monoclonal antibodies (Chemicon and Alexa Fluor 555–coupled goat anti-mouse immunoglobulin G (Invitrogen), and DNA was detected with Sytox Green (Invitrogen). Samples were mounted in Fluoromount G (Southern Biotech) with 2.5 mg/mL n-propyl gallate (Acros Organics).

Images were acquired on a Zeiss LSM 700 confocal laser scanning microscope (63×/1.40 oil immersion objective) in the University of Virginia Advanced Microscopy Core. A total of 5–10 slices with a thickness of 1 µm were captured as z stacks for 7–10 fields of view each. Z stacks were compressed using Zen 2012 (Zeiss), exported as TIF files, and imported into ImageJ (National Institutes of Health). The area of intact NETs per image was calculated as the mean gray value (MGV) of Sytox Green staining (using the “Measure” selection under the “Analyze” tab of ImageJ). The MGV is the sum of the gray values of all pixels in the selection, divided by the total number of pixels. The MGVs for each compressed stack were averaged per experiment. Results are expressed as the average MGV of at least 3 independent experiments per condition.

Luminol-Dependent Chemiluminescence

Reactive oxygen species generation from neutrophils was measured using luminol-dependent chemiluminescence as described elsewhere [23].

NET Bacterial Survival Assay

Neutrophils (1 × 10^6) were seeded in 24-well plates (Costar) and treated with 20 nM PMA for 15 minutes at 37°C in 5% CO2. One set of wells was treated with 10 µg/mL cytochalasin D (Sigma), a second was treated with cytochalasin D and 1 U of DNAse I, and the third was mock treated with RPMI 1640 medium, each for 15 minutes. Neutrophils were exposed to parent, Δnuc, or nuc-complement N. gonorrhoeae (multiplicity of infection, 1) for 1 hour. Well contents were serially diluted and plated for CFU enumeration. Bacterial survival is expressed as a percentage of the initial inocula per well. In specified experiments, DNAse I was added for 20 minutes after 1 hour of bacterial exposure.

Statistical Analyses

Two-tailed, unpaired Student t tests were used (Graph Pad Prism), with P values of <.05 considered statistically significant. Error bars are standard errors of the mean for the indicated number of independent experiments with different donors’ neutrophils.

RESULTS

NET Integrity in Response to N. gonorrhoeae

NET formation was examined in primary human neutrophils that were mock treated or exposed to PMA, parent N. gonorrhoeae, Δnuc N. gonorrhoeae, or nuc-complemented N. gonorrhoeae for 3 hours. Significantly greater NET content was measured in PMA-treated neutrophils, compared with untreated controls (Figure 1A). We observed significantly fewer NETs in neutrophils challenged with opacity protein–deficient parent or nuc-complemented N. gonorrhoeae of strain FA1090, compared with cells exposed to isogenic Δnuc N. gonorrhoeae (Figure 1A); similar observations were made with Opa-expressing strain 1291 parent and Δnuc N. gonorrhoeae (Supplementary Figure 1B) and at a multiplicity of infection of < 1 (data not shown). Neisseria gonorrhoeae–induced NETs contained neutrophil elastase, as well as LL-37 (Figure 1A and 1B).

The differences in NET integrity after exposure to Nuc-containing or Δnuc N. gonorrhoeae strains could be due to differences in NET induction and/or NET degradation. To test whether Δnuc N. gonorrhoeae has an enhanced ability to elicit NETs, we monitored NET integrity in neutrophils exposed to parent or Δnuc N. gonorrhoeae over time. NETs were released as early as 20 minutes after bacterial exposure. NET integrity was comparable between parent and Δnuc N. gonorrhoeae at times up to 1 hour, after which it decreased with parent N. gonorrhoeae but increased in Δnuc-exposed cells (Figure 2). Consistent with previous findings for this opa-deficient background [18], neither Nuc-containing nor Δnuc N. gonorrhoeae induced production of reactive oxygen species by neutrophils (Supplementary Figure 1C), indicating that effects on NET integrity were not due to differences in the neutrophil oxidative burst. Together, these results show N. gonorrhoeae induces rapid formation of NETs by human neutrophils and that NET integrity decreases over time with Nuc-containing N. gonorrhoeae.

Nuc Degrades NETs

To examine whether Nuc uses neutrophil DNA as a substrate, genomic DNA from primary human neutrophils was incubated with recombinant Nuc or, as a positive control, DNAse I. Nuc degraded neutrophil DNA in a concentration-dependent manner (Figure 3A) and was thermostable (Supplementary Figure 2A). Moreover, recombinant Nuc decreased the integrity of PMA-induced NETs (Figure 3B), as well as NETs elicited by Δnuc N. gonorrhoeae (Supplementary Figure 3A).

To examine whether Nuc-containing N. gonorrhoeae can degrade preformed NETs, PMA-treated neutrophils were exposed to parent or Δnuc N. gonorrhoeae. We observed significantly fewer intact NETs in the presence of parent N. gonorrhoeae, compared with Δnuc N. gonorrhoeae or no infection (Figure 4). Nuclease activity was present in the spent medium from cultures of parent N. gonorrhoeae and not Δnuc N. gonorrhoeae,
Figure 1. Neutrophil extracellular trap (NET) integrity in neutrophils exposed to Nuc-containing and Δnuc Neisseria gonorrhoeae. Primary human neutrophils were left untreated, stimulated with phorbol 12-myristate 13-acetate (PMA), or infected with parent N. gonorrhoeae, Δnuc N. gonorrhoeae, or nuc-complement N. gonorrhoeae for 3 hours. NETs were imaged by immunofluorescence for DNA (green) and neutrophil elastase (red; A) or for DNA and LL-37 (B). Area of Sytox-stained DNA within random fields of view was quantified for each condition as arbitrary units (AU) of the mean gray value. n = 4 experiments. *P < .025.
since conditioned medium from parent bacteria significantly reduced the integrity of NETs that were induced by PMA (Figure 5A) or by Δnuc Neisseria gonorrhoeae (Supplementary Figure 3B). Nuc activity was blocked by preincubating Nuc or parent N. gonorrhoeae supernatant with the thermonuclease inhibitor deoxythymidine 3',5'-diphosphate (Supplementary Figure 2) [15]. We conclude that N. gonorrhoeae releases Nuc, which cleaves human neutrophil DNA and NETs.

**Nuc Enhances N. gonorrhoeae Extracellular Survival in the Presence of Neutrophils**

Since NETs trap microorganisms and fewer intact NETs were observed with Nuc, we hypothesized that Nuc enhances bacterial escape from NETs. To test this hypothesis, neutrophils were stimulated with PMA. One set of samples underwent no further treatment, yielding neutrophils that release NETs and phagocytose bacteria. Another set was treated with cytochalasin D to inhibit phagocytosis. The third set received both cytochalasin D and DNAse I to degrade extracellular DNA. Neutrophils were then exposed to parent, Δnuc, or nuc-complement N. gonorrhoeae. Significantly fewer Δnuc N. gonorrhoeae were recovered from PMA-stimulated neutrophils relative to parent N. gonorrhoeae. While survival of parent and nuc-complement N. gonorrhoeae was significantly increased in the presence of cytochalasin D–treated neutrophils, compared with untreated neutrophils, Δnuc N. gonorrhoeae survival was unaffected, indicating that Δnuc N. gonorrhoeae is especially sensitive to extracellular killing by neutrophils. Addition of DNAse significantly increased recovery of Δnuc N. gonorrhoeae, implicating NETs in the survival defect of Δnuc N. gonorrhoeae after neutrophil challenge. CFUs of parent or complement N. gonorrhoeae exposed to cytochalasin D–treated neutrophils were not affected.

![Figure 2](image)

**Figure 2.** Neutrophil extracellular trap integrity over time in response to parent and Δnuc Neisseria gonorrhoeae. Neutrophils were exposed to parent or Δnuc N. gonorrhoeae. At the indicated times, samples were processed for immunofluorescence for neutrophil elastase (red) or DNA (green), and mean gray values were quantified as described in Figure 1. n = 3 experiments. *P < .05. Abbreviation: AU, arbitrary units.
Similar results were obtained when NETs were treated with recombinant Nuc instead of DNAse I and at bacteria to neutrophil ratios of 0.1 or 10 (data not shown).

Two possibilities could explain the reduced recovery of $\Delta nuc$ N. gonorrhoeae from NET-producing neutrophils: NETs are bactericidal for N. gonorrhoeae, or NETs trap N. gonorrhoeae and resist dispersion when CFUs are enumerated. To test between them, NET-producing neutrophils were treated with DNase I before versus after infection with $\Delta nuc$ N. gonorrhoeae. Adding DNase I after infection did not increase the CFU of $\Delta nuc$ N. gonorrhoeae (Figure 6B), implying the bacteria were being killed in NETs.

**Figure 3.** Nuc cleaves human neutrophil and neutrophil extracellular trap DNA. A, Human neutrophil DNA was incubated with increasing amounts of Nuc or DNAse I and separated on ethidium bromide–stained agarose gels. B, Phorbol 12-myristate 13-acetate–stimulated neutrophils were treated with indicated amounts of Nuc. Samples were processed for immunofluorescence for neutrophil elastase (red) or DNA (green), and mean gray values were quantified as described in Figure 1. $n = 3$ experiments. *$P < .05$. Abbreviation: AU, arbitrary units.

**Figure 4.** Nuc-containing Neisseria gonorrhoeae decreases the integrity of phorbol 12-myristate 13-acetate (PMA)–induced neutrophil extracellular traps. PMA-stimulated neutrophils were exposed to parent or $\Delta nuc$ N. gonorrhoeae. Samples were processed for immunofluorescence for neutrophil elastase (red) or DNA (green), and mean gray values were quantified as described in Figure 1. $n = 4$ experiments. *$P < .05$. Abbreviation: AU, arbitrary units.

by DNAse (Figure 6A). Similar results were obtained when NETs were treated with recombinant Nuc instead of DNase I and at bacteria to neutrophil ratios of 0.1 or 10 (data not shown).

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We conclude that NETs are made in response to N. gonorrhoeae and can capture and kill N. gonorrhoeae; however, Nuc degrades NETs, which enhances bacterial survival after neutrophil challenge.

**DISCUSSION**

The mechanisms by which N. gonorrhoeae survives after exposure to neutrophils remain incompletely understood. Here we show that N. gonorrhoeae stimulates the release of NETs from primary human neutrophils and that N. gonorrhoeae uses Nuc to degrade NETs and thereby evade NET-mediated killing. Nuc is sufficient to reduce NET integrity, whether delivered as recombinant protein, in conditioned medium from bacterial cultures, or by intact bacteria. Parent and Δnuc N. gonorrhoeae both elicit NETs from quiescent neutrophils, but the production of Nuc by the parent results in NET degradation. Moreover, Nuc-containing N. gonorrhoeae has a survival advantage after exposure to NET-producing neutrophils. These findings establish Nuc as a virulence factor in N. gonorrhoeae defense against innate immunity. Nuc could also contribute to survival of N. gonorrhoeae inside neutrophils, for instance by degrading DNA released by dead N. gonorrhoeae to provide live bacteria in the same phagosome with nucleotides. While all N. gonorrhoeae strains with genomes sequenced to date possess a full-length nuc gene, nuc is also present in N. meningitidis and some commensal neisseriae, suggesting additional contributions of Nuc to host colonization, including dispersal from biofilms [17].

Figure 5. Nuc activity in conditioned Neisseria gonorrhoeae culture supernatants degrade neutrophil extracellular traps (NETs). Neutrophils were treated with phorbol 12-myristate 13-acetate (PMA) to stimulate NET formation. Neutrophils were then treated with conditioned medium from parent or Δnuc N. gonorrhoeae, treated with DNase I, or left untreated. Samples were processed for immunofluorescence for neutrophil elastase (red) or DNA (green), and mean gray values were quantified as described in Figure 1. n = 3 experiments. *P<.05. Abbreviation: AU, arbitrary units.

Figure 6. Nuc enhances Neisseria gonorrhoeae survival in the presence of neutrophils releasing neutrophil extracellular traps (NETs). A, Neutrophils were treated with phorbol 12-myristate 13-acetate (PMA) to induce NETs and then mock treated (left), treated with cytochalasin D (middle), or treated with cytochalasin D plus DNase I (right). Parent N. gonorrhoeae (black bars), Δnuc N. gonorrhoeae (white bars), or nuc-complement N. gonorrhoeae (nuc::nuc) (hatched bars) were then added. Bacterial survival is expressed as the percentage of the initial inocula. n = 6 experiments; *P<.05. B, PMA and cytochalasin D–treated neutrophils were exposed to DNase I (DNase pretreatment) or not, then challenged with Δnuc N. gonorrhoeae. In the nonpretreated samples, neutrophils were then exposed to DNase I (DNase posttreatment) or an equal volume of medium (medium control). Colony-forming units (CFU) were then enumerated as above. There was no significant difference between the latter two conditions. n = 3 experiments. Abbreviation: NS, not significant.
Species of *Streptococcus*, *Staphylococcus*, *Vibrio*, and *Leishmania* express nucleases that aid in escape from NETs, to promote pathogen survival and spread [24–28]. We now add *N. gonorrhoeae* to this list, with 3 pieces of evidence to show that *N. gonorrhoeae* Nuc can degrade NETs. First, Nuc degrades human neutrophil genomic DNA. Second, fewer intact NETs are observed when Nuc is added to NET-producing neutrophils. Third, conditioned supernatant from parent *N. gonorrhoeae* reduces NET integrity. *Neisseria gonorrhoeae* Nuc shares several characteristics with *S. aureus* thermonuclease, including a high degree of sequence similarity, with conservation of several identical residues in predicted active-site locations [17], heat-stable activity, and inhibition by deoxynthymidine 3′,5′′-diphosphate.

Given the conservation of Nuc in *N. gonorrhoeae* and the immunogenicity of *S. aureus*, this may be due to meningococcal-specific virulence factors, such as capsular polysaccharide [31]. We hypothesize that NETs kill *N. gonorrhoeae* by placing the bacteria in proximity to NET-associated cationic antimicrobial proteins. These components include LL-37 and cathepsin G, which have antigonococcal activity in vitro [32–35] and, for cathepsin G, in neutrophil phagolysosomes [13].

These results lead us to revise our model for the interplay between *N. gonorrhoeae* and neutrophils during human infection. Neutrophils that are recruited to the infected mucosa are exposed to *N. gonorrhoeae* and cytokines that promote neutrophil activation. *Neisseria gonorrhoeae* has evolved a variety of mechanisms for resisting clearance by neutrophils, including expression of protective gene products, delay of phagosome maturation, and blockade of the oxidative burst [5, 13]. In this highly inflamed environment, it is likely that some neutrophils release NETs. While NETs contain species with antigonococcal activity, *N. gonorrhoeae* uses Nuc to help escape them. Thus, Nuc-mediated NET degradation contributes to the strategies used by *N. gonorrhoeae* to avoid killing inside and outside of neutrophils, thereby enabling the persistence of *N. gonorrhoeae* in its obligate human hosts.

**Supplementary Data**

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

**Notes**

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