

Role of the Gonococcal Neisserial Heparin Binding Antigen in Microcolony Formation, and Serum Resistance and Adherence to Epithelial Cells

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The sexually transmitted infection gonorrhoea is on the rise worldwide and an increased understanding of the mechanisms of colonization and pathogenesis of *Neisseria gonorrhoeae* is required to aid development of new treatment and prevention strategies. In the current study, we investigate the neisserial heparin-binding antigen (NHBA) of *N. gonorrhoeae* and confirm its role in binding to several glycans, including heparin, and identify interactions of NHBA with both gonococcal and host cells. Furthermore, we report that a gonococcal *nhba* mutant displays decreased cell aggregation and microcolony formation, as well as reduced survival in human serum and reduced adherence to human cervical and urethral epithelial cells, relative to the wild-type strain. These data indicate that the gonococcal NHBA contributes to several aspects of the colonization and survival of *N. gonorrhoeae* and may be a target for new antimicrobial or vaccines.

Keywords. NHBA; neisserial heparin-binding Antigen; glycan; heparin; aggregation; microcolony; *Neisseria gonorrhoeae*; gonococcus; vaccine antigen; adherence; serum resistance.

Neisseria gonorrhoeae is an obligate human pathogen that causes approximately 106 million cases of the sexually transmitted infection gonorrhoea worldwide each year [1]. *N. gonorrhoeae* infects human mucosal surfaces, resulting in various clinical outcomes including local symptomatic disease (eg, cervicitis, urethritis, conjunctivitis), and occasionally disseminated disease [2]. Asymptomatic infections of the genital tract, rectum, or pharynx are common, and untreated genital tract infections can lead to severe sequelae, such as pelvic inflammatory disease, adverse pregnancy outcomes, neonatal complications, infertility, and increased risk of human immunodeficiency virus acquisition (reviewed in [3]).

The control of *N. gonorrhoeae* is a major public health challenge owing to the emergence of multidrug-resistant strains [4, 5]. Gonococcal strains with high-level resistance to expanded-spectrum cephalosporins, ceftriaxone and cefixime, as well as

azithromycin and all other antibiotics routinely used to treat gonorrhoea, have been identified globally [6, 7]. This highlights the need for investigation of gonococcal pathogenesis to aid development of novel therapeutics and a vaccine.

The neisserial heparin-binding antigen (NHBA) is present in the 4 component meningococcal serogroup B vaccine (4CMenB; Bexsero) licensed to protect against invasive disease caused by *Neisseria meningitidis* [8], which is closely related to *N. gonorrhoeae*. The gonococcal homologue of NHBA is surface exposed and highly conserved in *N. gonorrhoeae* strains (>93% identity), shares 67% identity [9] with the 4CMenB NHBA, and is recognized by human serum samples from people vaccinated with 4CMenB [10].

The meningococcal NHBA has most extensively been studied in strain MC58 (expresses NHBA-3) and was named based on its ability to bind the glycosaminoglycan (GAG) heparin via an arginine-rich region (Arg region), and NHBA binding to heparin increases meningococcal resistance to serum [11] and interactions with heparan sulfate mediates binding to epithelial cells [12]. NHBA binds several other glycans, with the highest affinity binding to chondroitin sulfate [13]. The meningococcal NHBA is the target of several proteases, including human lactoferrin [11], kallikrein [14] and C3-convertase [15], as well as meningococcal NaIP [11]. NaIP cleaves NHBA after the arginine-rich region, and it has been speculated that hypervirulent strains of *N. meningitidis* that express NaIP release a NHBA fragment that increases vascular permeability [16]. NHBA-2 also has increased expression at lower temperatures (32°C vs 37°C) [17] and plays a role in biofilm formation [18]. The gonococcal NHBA has not yet been characterized;

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however, *N. gonorrhoeae* does not express NalP [19], and its NHBA has a truncated Arg region [9] indicating that it may play a different role in *N. gonorrhoeae* than in *N. meningitidis*. In the current study, we examined the function of the gonococcal NHBA in order to describe its role in pathogenesis and support its potential use as a gonococcal therapeutic target or vaccine candidate.

METHODS

N. Gonorrhoeae Growth and Recombinant Techniques

N. gonorrhoeae 1291 was cultured on GC agar (Oxoid) or GC broth with 1% (vol/vol) IsoVitaleX (Becton Dickinson) at 32°C or 37°C, 5% carbon dioxide [20]. The *nhba::kan* mutant (Δ NHBA) and complemented (Δ NHBA_C) strains were generated as described in [Supplementary Table 1](#). Expression of pilin, opacity (Opa) protein, porin, and lipooligosaccharide (LOS) was similar in the wild-type (WT), Δ NHBA, and Δ NHBA_C strains ([Supplementary Figure 1](#)). Recombinant His-tagged NHBA was expressed and purified as described elsewhere [10].

Western Blot Analysis, Enzyme-Linked Immunosorbent Assay, and Flow Cytometry

Western blot analysis of *N. gonorrhoeae* whole-cell lysates was performed as described elsewhere [21], with mouse anti-NHBA (see Supplementary Methods) and rabbit anti-NGAG_01228 [21]. Enzyme-linked immunosorbent assay (ELISA) of recombinant NHBA binding to whole-cell *N. gonorrhoeae* was performed after 30-minute incubation at room temperature, using horseradish peroxidase-conjugated His-tag antibody (Thermo) and following standard protocols [10, 22]. Flow cytometry was performed using a CyAn ADP cytometer (Beckman Coulter), as described elsewhere [21, 23], with bacteria (approximately 10^8 colony-forming units [CFUs]), anti-NHBA (1:200, 30 minutes), and Alexa Fluor 488-conjugated anti-mouse immunoglobulin G (1:200, 1 hour; Thermo). Binding of fluorescein isothiocyanate-labeled NHBA (100 μ g/mL) to *N. gonorrhoeae* (approximately 10^7 CFUs) or to E6/E7-transformed primary human cervical epithelial (tCX) and urethral epithelial (tUEC) cells (approximately 5×10^5 cells) was measured after incubation for 20 minutes at 37°C.

Microscopy

Fluorescent microscopy was used to measure the interaction of NHBA (100 μ g/mL) incubated with tCX cells (cultured on glass coverslips to full confluence) at 37°C for 20 minutes. Cells were washed 3 times to remove unbound proteins and fixed in formaldehyde (2.5% for 15 minutes). NHBA-542 (referred to herein as NHBA_{Ng}) was detected using anti-NHBA (1:1000) [10] and Alexa Fluor 488-conjugated anti-mouse immunoglobulin G (1:200; Thermo). Cells were counterstained with Alexa Fluor 568 Phalloidin (Thermo) and DAPI. Glass coverslips were mounted on microscope slides using ProLong Gold Antifade

Mountant (Thermo), images were captured on a Nikon A1R confocal microscope, and data were analyzed using NIS-Elements software version 1.2.3 (Nikon).

Gonococcal microcolony formation was investigated using tUEC cells incubated with approximately 1×10^6 CFUs at 37°C for 5 hours. Cell monolayers were washed 3 times (with Hanks' balanced salt solution) to remove nonadherent bacteria and fixed for 30 minutes, and scanning electron microscopy (JCM-5000 NeoScope; JEOL) was performed as described elsewhere [24].

Glycan Binding

Glycan array experiments were performed with recombinant NHBA (1 μ g), as described elsewhere [13, 25]. Surface plasmon resonance (BIAcore T200) was performed with NHBA (100 μ g/mL) immobilized on flow cells 2–4 by amine coupling on series S CM5 sensor chips (GE Healthcare), as described elsewhere [13, 22]. Single-cycle kinetics was used to calculate the affinity (dissociation constant [K_D]) of interactions with glycans run in 1:5 dilution series at concentrations between 100 μ mol/L and 1 nmol/L

Functional Assays

Resistance of *N. gonorrhoeae* to serum-mediated killing was tested as described elsewhere [21], with approximately 10^4 CFUs incubated in 10% normal human serum for 60 minutes. Bacteria were preincubated with 6 μ mol/L heparin for 30 minutes where indicated.

Gonococcal adherence to tCX and tUEC cells was assayed with approximately 10^5 CFUs for 1 hour as described elsewhere [23]. For adherence-blocking assays, cells were pretreated with recombinant gonococcal NHBA (1–100 μ g/mL) or peanut agglutinin (PNA; 100 μ g/mL; negative control that does not bind tCX [23]), before being infected with *N. gonorrhoeae* for 10 minutes. Adherence and serum survival assays were performed in triplicate on 3 occasions, and statistical analysis performed with analysis of variance and Student *t* tests.

RESULTS

Sequence Features and Expression of Gonococcal NHBA

The main NHBA variant expressed by *N. gonorrhoeae* strains is NHBA-542, which is present in >40% of gonococcal isolates in the PubMLST database, including strain 1291 [10]. NHBA_{Ng} is 426 amino acids and contains sequence features similar to those described for the well-characterized NHBA-3 from *N. meningitidis* strain MC58 (referred to herein as NHBA_{Nm}), including a lipobox motif and a polyglycine stretch in the N-terminal and an arginine-rich region in the central region of the protein ([Figure 1A](#)). However, there are several differences between NHBA_{Ng} and NHBA_{Nm} owing to insertions and deletions ([Figure 1B](#)). The N-terminal half of the gonococcal *nhba* gene has a 63-amino acid deletion compared with NHBA-3, and this is consistent in

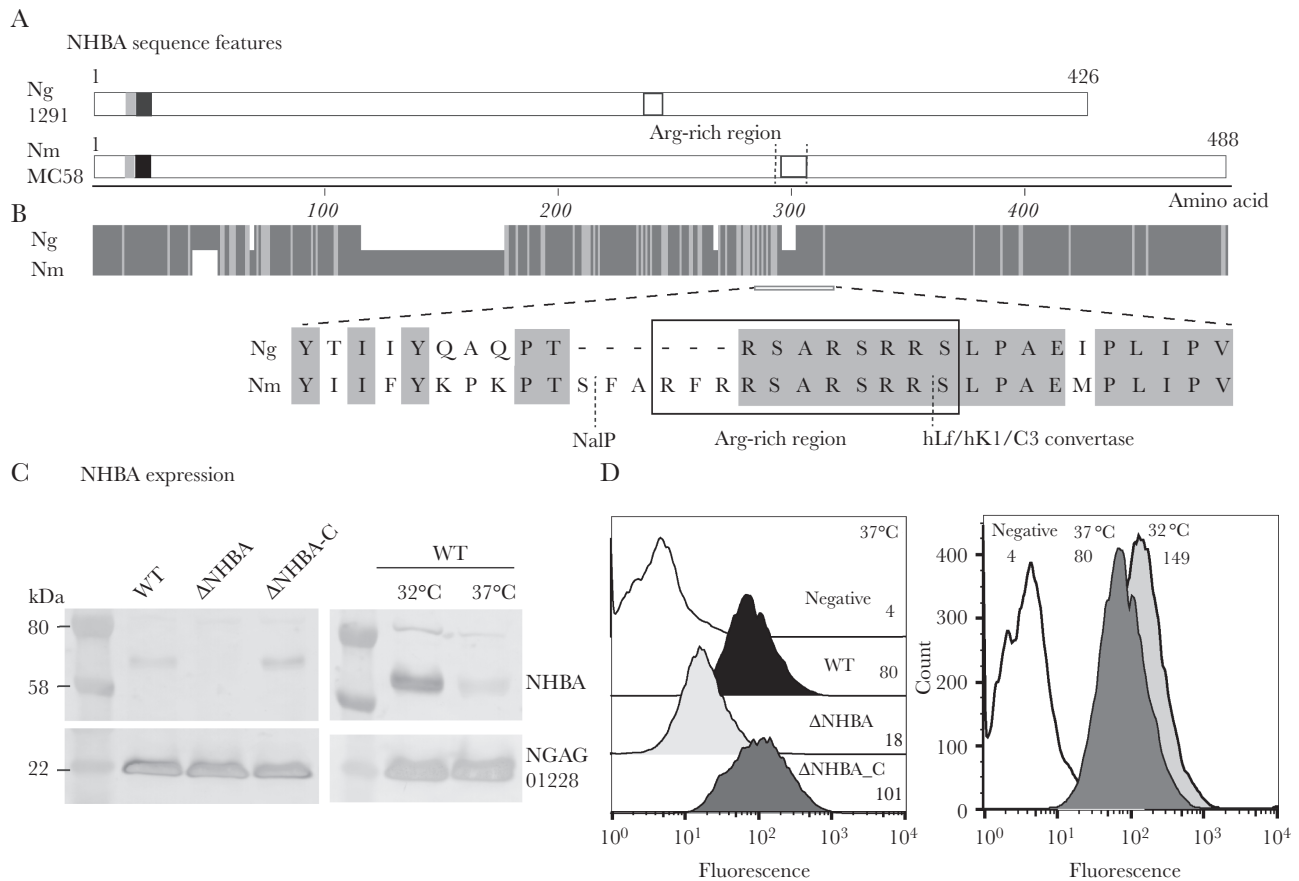


Figure 1. Neisserial heparin-binding antigen (NHBA) sequence features and expression. *A*, Schematic of *Neisseria gonorrhoeae* (Ng) strain 1291 NHBA and *Neisseria meningitidis* (Nm) strain MC58 NHBA proteins is shown, with the lipobox motif (gray box) and glycine stretch (black box) shown in the N-terminal, and the arginine-rich region (white box) shown in the central region of NHBA. The amino acid length of each protein is shown on the right. *B*, The *N. gonorrhoeae* and *N. meningitidis* NHBA proteins were aligned using ClustalW in MacVector software (version 17.0) (see Supplementary Methods). Identical amino acids are shown as dark gray vertical lines, mismatches light gray lines, and gaps as white. The amino acid sequences of the arginine-rich region in the *N. gonorrhoeae* and *N. meningitidis* NHBA proteins (boxed) and its flanking sequences are shown, with identical amino acids shaded gray and mismatches shown in white. The cleavage site of the meningococcal NalP upstream of the Arg-rich region, and human lactoferrin (hLf), kallikrein (hK1) and C3 convertase are shown. *C*, Western blot of whole-cell lysates of the *N. gonorrhoeae* 1291 wild-type (WT), NHBA knockout (Δ NHBA), and complemented (Δ NHBA_C) strains grown at 37°C, using polyclonal anti-NHBA antibodies. Up-regulation of NHBA expression in the WT grown at 32°C versus 37°C is also shown. The periplasmic protein NGAG_01228 is shown as a loading control. *D*, Flow cytometry of whole cells of *N. gonorrhoeae* WT, Δ NHBA and Δ NHBA_C strains grown at 32°C and 37°C with the expression of NHBA on the cell surface being confirmed using polyclonal anti-NHBA antibody. The negative control is WT with secondary antibody only. Values represent geometric mean fluorescence.

the major gonococcal variants (Supplementary Figure 2). The Arg region of NHBA_{Ng} is truncated compared with NHBA_{Nm}. This region is highly conserved between *N. meningitidis* strains [11] and between the major *N. gonorrhoeae* NHBA variants (Supplementary Figure 2; the variants shown are present in 94% of 3068 isolates [10]).

To characterize NHBA_{Ng}, a recombinant His-tagged protein NHBA_{Ng} was generated in *E. coli* and polyclonal anti-NHBA antibodies raised in mice. In addition, an isogenic mutant of NHBA was generated by insertion of a kanamycin resistance cassette into the *nhba* gene in *N. gonorrhoeae* strain 1291 (Δ NHBA), and this mutant was complemented by reintroducing a single copy of *nhba* into the genome in *trans* (Δ NHBA_C). Western blot analysis of whole-cell lysates confirmed expression of NHBA in the WT strain, with a single

band detected between 58 and 80 kDa by anti-NHBA sera. The complemented strain expressed levels of NHBA similar to those in the WT, whereas no NHBA expression was detected in Δ NHBA (Figure 1C and Supplementary Figure 1). NHBA was also detected on the surface of whole-cell *N. gonorrhoeae* WT and Δ NHBA_C strains by flow cytometry (Figure 1D). Growth of *N. gonorrhoeae* strains to midlog phase at 32°C and 37°C revealed that NHBA_{Ng} expression is temperature regulated, with higher expression at lower temperatures (Figure 1C and Supplementary Figure 3).

Involvement of Gonococcal NHBA in Cell Aggregation and Microcolony Formation

To investigate the role of NHBA_{Ng} in growth in vitro, the *N. gonorrhoeae* 1291 WT, Δ NHBA, and Δ NHBA_C strains

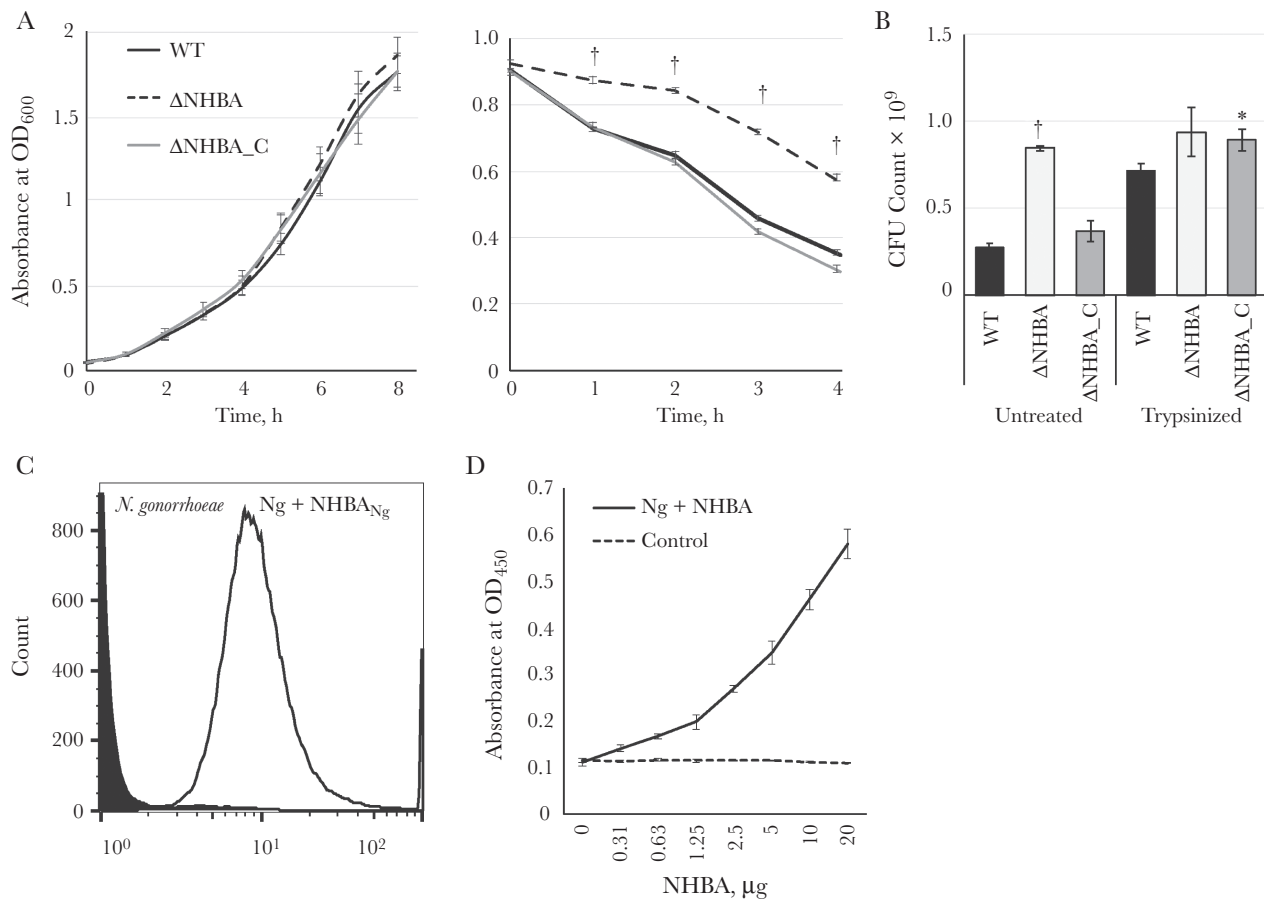


Figure 2. Gonococcal neisserial heparin-binding antigen (NHBA) is involved in cell aggregation. *A*, Growth and settling curves of *Neisseria gonorrhoeae* 1291 wild-type (WT), NHBA knockout (Δ NHBA), and complemented (Δ NHBA_C) strains in gonococcal broth, with absorbance measured at an optical density at 600 nm (OD₆₀₀). *B*, *N. gonorrhoeae* colony-forming units (CFUs) per milliliter in a culture at an OD₆₀₀ of 1, before and after trypsinization (0.25% trypsin for 5 minutes). The CFU counts of WT and Δ NHBA_C strains increased 2.6-fold and 2.4-fold, respectively, after trypsin treatment (both $P < .001$), whereas the Δ NHBA CFU count was not affected ($P = .31$). * $P < .05$; † $P < .001$ (comparison to WT). *C*, Flow cytometric analysis of recombinant *N. gonorrhoeae* NHBA (NHBA)_{Ng} binding to whole-cell *N. gonorrhoeae* (black represents *N. gonorrhoeae* only; white, *N. gonorrhoeae* plus labeled NHBA_{Ng}). *D*, Whole-cell enzyme-linked immunosorbent assay titration curve showing binding of NHBA_{Ng} to *N. gonorrhoeae* (black line). The antibody-only control curve (dotted line) indicates the absence of nonspecific interactions between whole-cell *N. gonorrhoeae* and the His-tag antibody.

were grown with gonococcal broth and agar. All strains had equivalent growth rates and maximal growth levels in terms of optical density (OD); however, the Δ NHBA mutant strain had significantly reduced settling rates compared with the WT and Δ NHBA_C strains (Figure 2A). Furthermore, when OD-equalized samples (OD at 600 nm [OD₆₀₀], 1) were plated onto gonococcal agar, the Δ NHBA strain had approximately 3-fold higher viable CFU counts compared with the WT or Δ NHBA_C strains (Figure 2B).

Treatment of these samples with trypsin resulted in equalized CFU counts for all 3 strains (WT and Δ NHBA_C countable CFUs increased 2.6- and 2.4-fold, respectively, whereas the Δ NHBA CFU count was not affected) (Figure 2B), indicating that the phenotype was due to cell aggregation rather than a defect in cell separation. Gram stain analysis of the 3 strains with or without trypsin treatment confirmed the presence of cell aggregates in the untreated WT and Δ NHBA_C strains (Supplementary Figure 4A). Furthermore, Western blot analysis indicated that trypsin

treatment digested NHBA from the bacteria surface but did not alter a periplasmic control protein (Supplementary Figure 4B). After this finding, the volume of Δ NHBA sample used in subsequent experiments was adjusted to equalize the CFU counts. Gonococcal pili and Opa proteins have been previously implicated in formation of bacterial aggregates, and phase-contrast microscopy confirmed that the WT, Δ NHBA, and Δ NHBA_C colonies were morphologically identical, in terms of piliation and opacity.

To determine whether NHBA_{Ng} directly interacts with the gonococcal surface to facilitate aggregation, we used flow cytometry and ELISA with recombinant NHBA_{Ng}. Flow cytometric analysis showed binding of the fluorescein isothiocyanate-labeled recombinant NHBA_{Ng} to whole-cell *N. gonorrhoeae* (Figure 2C). This was confirmed using whole-cell ELISA, in which recombinant NHBA_{Ng} bound *N. gonorrhoeae* in a concentration-dependent manner (Figure 2D).

To further study the role of NHBA in bacteria-bacteria interactions and in the formation of gonococcal aggregates, we

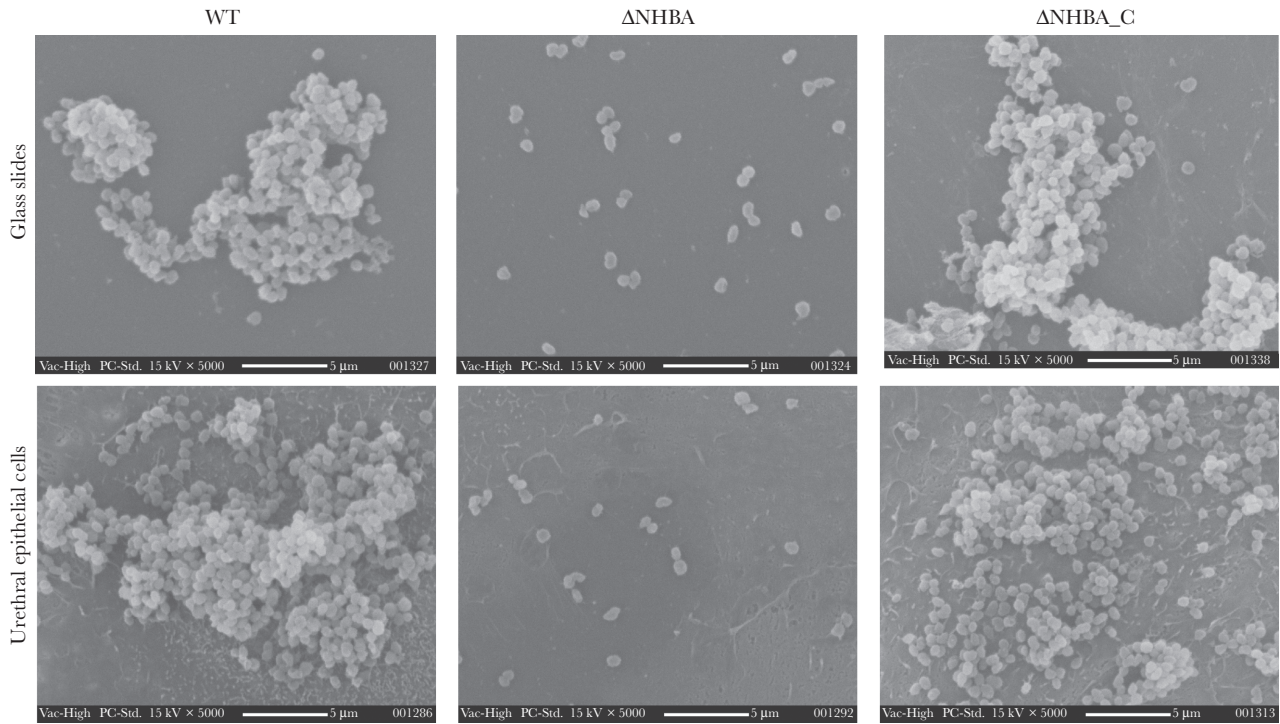


Figure 3. Gonococcal neisserial heparin-binding antigen (NHBA) is involved in microcolony formation. Scanning electron microscopy shows *Neisseria gonorrhoeae* 1291 wild-type (WT), NHBA knockout (Δ NHBA), and complemented (Δ NHBA_C) strains grown for 5 hours on glass sides (*top panel*) or on human urethral epithelial cell monolayers on glass slides (*bottom panel*). Aggregates and microcolonies can be seen for the WT and Δ NHBA_C strains, whereas the Δ NHBA strain is seen as single colonies or diplococci. Images were acquired at a magnification of $\times 5000$, and the scale bar at the bottom of each box represents 5 μ m.

examined the ability of Δ NHBA to form microcolonies. Unlike the WT or Δ NHBA_C strains, Δ NHBA was unable to form microcolonies on the surface of glass coverslip slides or tUEC monolayers after 5-hour growth (Figure 3). Biofilm assays were also performed to investigate whether the self-association properties of NHBA_{Ng} play a role in the establishment of gonococcal biofilm. However, under static conditions over 24–26 hours, no difference in biofilm formation was observed for the WT, Δ NHBA, and Δ NHBA_C strains (data not shown).

NHBA_{Ng} Binding to Glycans With High Affinity

The glycan-binding profile of the gonococcal NHBA was determined using glycan array analysis with arrays that display 368 structures representative of glycans found on human cells (including isomers and/or glycans that have similar structure but differ in chain length, chemical linkage or spacer size). NHBA_{Ng} bound to 39 glycan structures on the array (Supplementary Figure 5 and Supplementary Table 2), including the GAGs heparin, heparan sulfate, and chondroitin sulfate. NHBA_{Ng} also bound multiple structures that contain a lacto-*N*-biose and *N*-acetylglucosamine core structure (ie, lacto-*N*-neotetraose [LNnT]) including their sialylated and fucosylated variants (ie, sLeX), as well as a limited set of *N*-acetylglucosamine, *N*-acetylgalactosamine, glucosyl, and mannosyl glycans.

To characterize the affinity of NHBA_{Ng}-glycan interactions, surface plasmon resonance analysis was performed using

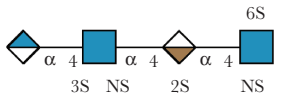
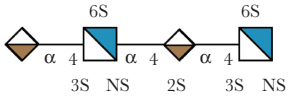

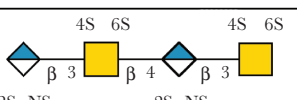

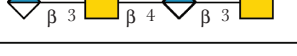


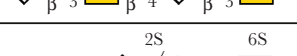


selected GAGs (Figure 4A) and non-GAG glycans (Figure 4B) that were bound on the array. The highest calculated affinity of all tested interactions was with heparin (K_D , 4.4 nmol/L), followed by chondroitin sulfate (K_D , 73 nmol/L). GAG structures are highly heterogeneous, comprising repeating polysaccharides with various sulfation patterns. To determine whether NHBA_{Ng} preferentially binds glycans with specific sulfation configuration, we performed experiments with 3 types of chondroitin sulfate (A, B, and C). NHBA_{Ng} bound only chondroitin sulfate C (chondroitin 6-sulfate) and no concentration-dependent binding was observed for chondroitin sulfate A (chondroitin 4-sulfate) or chondroitin sulfate B (dermatan sulfate).

We also show that NHBA_{Ng} binds heparan sulfate, but with lower affinity (K_D , 2.79 μ mol/L). In terms of non-GAG glycans, NHBA_{Ng} binds α 2–6 sialylated pentasaccharide—LSTc (K_D , 0.24 μ mol/L)—with higher affinity than its isomer LSTb (K_D , 2.26 μ mol/L), and a nonsialylated variant of LNnT (K_D , 4.89 μ mol/L). Furthermore, NHBA_{Ng} has 5-fold greater affinity for Lewis X (K_D , 0.68 μ mol/L) than for sialyl Lewis X (K_D , 3.65 μ mol/L). No concentration-dependent binding was observed for hyaluronan (a nonsulfated GAG) or H-disaccharide, which were not bound by NHBA_{Ng} on the glycan array and were used as negative controls.





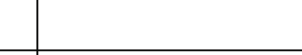
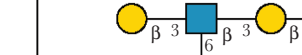
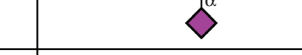
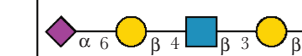

Gonococcal NHBA Binding to Epithelial Cells

NHBA_{Nm} binds to epithelial cells through its Arg region via interactions with heparan sulfate proteoglycans [12]. To

A

Name	Structure	K_D , $\mu\text{mol/L}$	
		NHBA _{Ng}	NHBA _{Nm}
Heparan sulfate		2.79 (1.83)	1.36 (0.2)
Heparin		0.0044 (0.0017)	0.052 (0.026)
Hyaluronan		NB	0.13 (0.03)
Chondroitin sulfate A, C, D mix		0.073 (0.021)	0.0052 (0.0024)
A		NB	NB
B		NB	NB
C		2.43 (1.04)	71.8 (16.9)
Chondroitin disaccharide $\Delta\text{Di-disD}$		7.54 (2.87)	0.0095 (0.0029)
$\Delta\text{Di-disB}$		5.11 (2.98)	0.52 (0.045)
$\Delta\text{Di-trisS}$		4.58 (3.20)	NB
$\Delta\text{Di-UA2S}$		4.38 (2.88)	NB

B

Name	Structure	K_D , $\mu\text{mol/L}$	
		NHBA _{Ng}	NHBA _{Nm}
H-disaccharide		NB	NB
Lewis X (CD15)		0.68 (0.16)	NB
Sialyl Lewis X (CD15s)		3.65 (2.62)	NB
LSTb		2.26 (1.00)	NB
LSTc		0.24 (0.09)	NB
Lacto-N- neotetraose (LNnT)		4.89 (1.71)	NB
β -Glc6P		4.72 (2.67)	NB
Hexaacetyl chitohexaose		8.93 (4.71)	NB
α 1-2 Mannobiose		2.99 (1.21)	NB

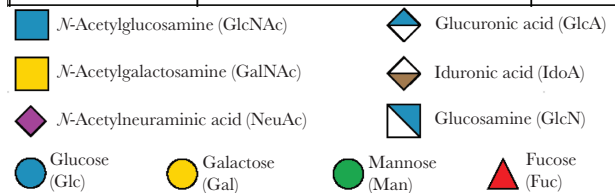


Figure 4. Gonococcal neisserial heparin-binding antigen (NHBA) binds to several glycans with high affinity. Surface plasmon resonance analysis of recombinant *N. gonorrhoeae* recombinant *N. gonorrhoeae* NHBA (NHBA_{Ng}) (binding to glycosaminoglycans (GAGs) (A) and non-GAG glycans (B)). The name, structure and sulfation patterns (S) of each glycan is shown, along with the dissociation constant (K_D) of NHBA_{Ng} binding to each glycan reported as mean (standard deviation). K_D values are also shown for recombinant *N. meningitidis* NHBA (NHBA_{Nm}) interactions [13], to enable comparison. NHBA_{Nm} has higher affinity for β -Glc6P (K_D , 0.056 [0.025] $\mu\text{mol/L}$). Results were analyzed using BIAcore T200 software 2.0.2. Abbreviations: NB, no concentration-dependent binding; NS, non sulfated.

investigate whether NHBA_{Ng} also interacts with epithelial cells, confocal microscopy and flow cytometric analysis were performed with recombinant NHBA_{Ng} and tCX and tUEC cells. For confocal microscopy, recombinant NHBA_{Ng} was incubated with tCX cells and detected using mouse anti-NHBA primary and Alexa Fluor 488 secondary antibodies. Binding of NHBA_{Ng} to tCX cells was observed (Figure 5A, top left) with the protein signal localized to the surface of cells (Figure 5A, bottom left). No nonspecific binding of the primary (Figure 5A, top right) or secondary (Figure 5A, bottom right) antibody to tCX cells was observed. Flow cytometric analysis further confirmed that NHBA_{Ng} binds both tCX and tUEC cells (Figure 5B).

Contribution of NHBA to Gonococcal Serum Survival and Adherence to Epithelial Cells

To investigate the functional role of NHBA_{Ng} interactions with glycans, gonococcal cells and human epithelial cells, we conducted serum survival and epithelial cell adherence assays. Serum survival assays performed with the WT, ΔNHBA , and ΔNHBA_C strains in 10% normal human serum (sublethal serum concentration for the WT strain) indicated that the ΔNHBA strain had approximately 3-fold reduced survival relative to the WT and ΔNHBA_C strains (Figure 6A). Pretreatment of gonococci with heparin increased survival of the ΔNHBA mutant strain to a level comparable to those of the WT and ΔNHBA_C strains.

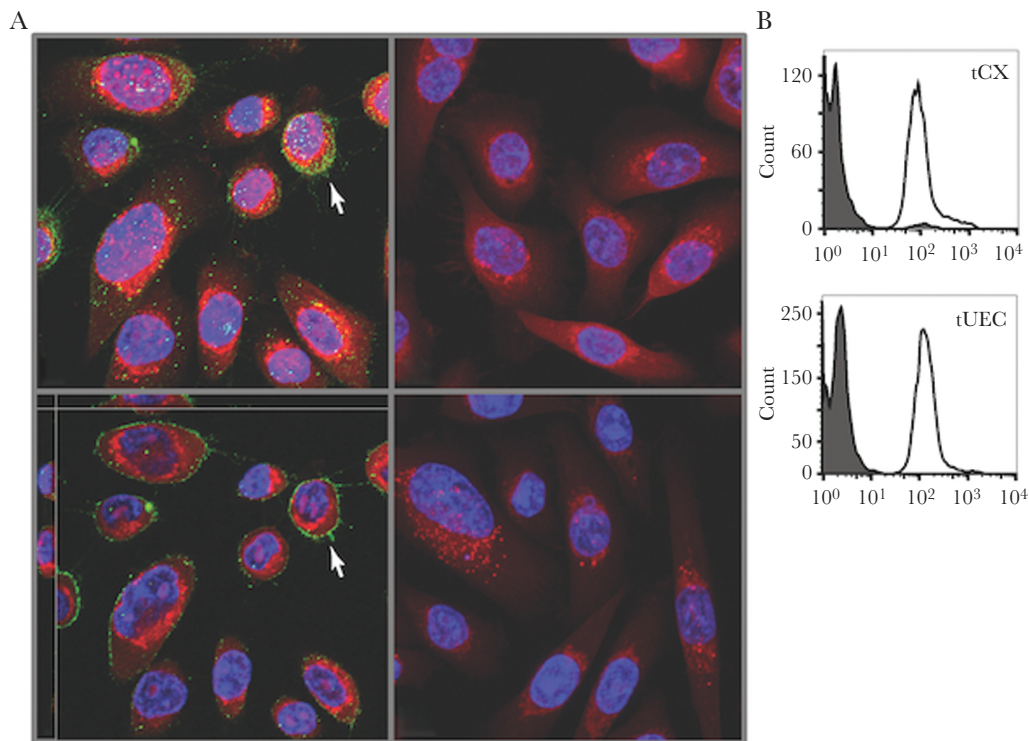


Figure 5. Gonococcal recombinant neisserial heparin-binding antigen (NHBA_{Ng}) binds to epithelial cells. *A*, Confocal fluorescent images acquired under $\times 40$ magnification of NHBA_{Ng} binding to cervical epithelial (tCX) cells. *Left*, Extended focus (*top left*) and xyz (*bottom left*) images showing cross-sections of cells. *Right*, Antibody control (no NHBA_{Ng}) images of untreated cells (*top right*) and cells treated with Recombinant *N. gonorrhoeae* NHBA (NHBA_{Ng}) and secondary antibody (*bottom right*). Arrows indicate protein localizing on the cell surface. *B*, Flow cytometric analysis of binding of recombinant NHBA_{Ng} to human tCX and urethral epithelial (tUEC) cells.

To investigate the role of NHBA_{Ng} in *N. gonorrhoeae* infection, we conducted in vitro infection assays with tCX and tUEC cells and the WT, Δ NHBA, and Δ NHBA_C strains. For infection assays with epithelial cells, the Δ NHBA mutant had 11-fold and 12-fold reduced adherence of tCX and tUEC cells, respectively, relative to the WT strain (Figure 6B). We also conducted adherence assays with cells that were pretreated with either recombinant NHBA_{Ng} or negative control protein PNA. Gonococcal adherence to NHBA_{Ng}-treated cells was reduced in a concentration-dependent manner (2.5- and 1.7-fold with 100 and 10 μ g/mL of NHBA_{Ng}, respectively), whereas treatment of cells with 100 μ g/mL PNA, a negative control that does not bind cells, had no effect on adherence (Figure 6C).

DISCUSSION

The sexually transmitted infection gonorrhoea is a growing public health concern owing to rising rates of infection and increasing antimicrobial resistance. *N. gonorrhoeae* transmission, colonization, and pathogenesis are complex, multifactorial processes (reviewed in [26]), and an increased understanding of all stages of gonococcal infection is required to aid development of new treatment and prevention strategies. In the current study, we characterized the interactions of gonococcal NHBA with glycans, *N. gonorrhoeae*, and human epithelial cells,

highlighting its involvement in microcolony formation, resistance to human serum, and adherence to epithelial cells.

NHBA was first identified in *N. meningitidis* during development of the meningococcal serogroup B vaccine 4CMenB [8, 11–15]. Despite a relatively high level of sequence identity between the gonococcal and meningococcal NHBA proteins (approximately 67% [10]), several differences between the NHBA sequences and the conditions encountered by the pathogenic *Neisseria* species prompted a detailed analysis of NHBA in *N. gonorrhoeae*. NHBA is more conserved in *N. gonorrhoeae* than in *N. meningitidis* [10], and in the current study we confirmed that the 63-amino acid deletion in the N-terminus and the truncated Arg region in *N. gonorrhoeae* are conserved in all major gonococcal NHBA variants.

In our glycan array analysis, recombinant NHBA_{Ng} bound to 39 glycans, including GAGs, such as heparin, heparan sulfate, and chondroitin sulfate. Our group previously showed that NHBA_{Nm} interacts with 28 glycans [13], and our surface plasmon resonance analysis confirmed that NHBA_{Ng} and NHBA_{Nm} bind ≥ 4 glycans in common (heparin, heparan sulfate, chondroitin sulfate, Glc-6P). However, a key difference between the proteins is that NHBA_{Ng} has higher binding affinity for heparin, and lower affinity for chondroitin sulfate and glucose 6-phosphate than NHBA_{Nm}. This is probably due to differences

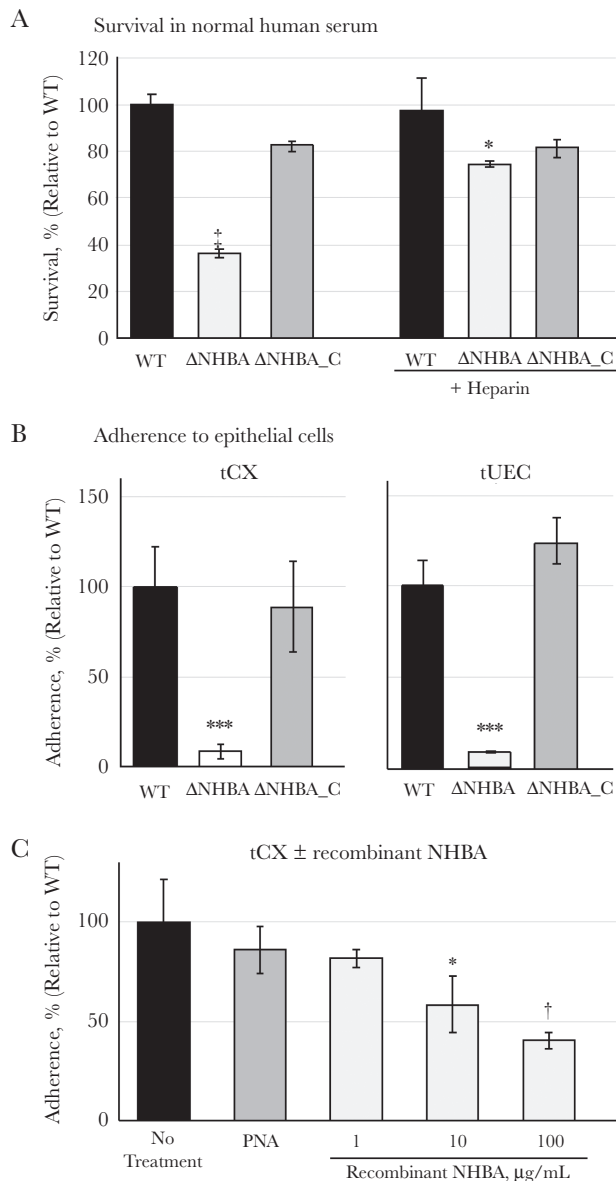


Figure 6. Gonococcal neisserial heparin-binding antigen (NHBA) contributes to survival in human serum and adherence to human epithelial cells. *A*, Survival of *Neisseria gonorrhoeae* 1291 wild-type (WT), NHBA knockout (Δ NHBA), and complemented (Δ NHBA_C) strains after 60 minutes in 10% (vol/vol) normal human serum. ‘+ Heparin’ indicates that bacteria were preincubated with heparin prior to addition of normal human serum. Data represent the average percentage survival for triplicate samples as a percentage of the inoculum and are shown relative to the WT (results for the WT strain, set at 100%, are 5.5×10^4 colony-forming units [CFUs]). There was no significant difference in survival of the WT in serum according to the absence or presence of heparin. *B*, Adherence of *N. gonorrhoeae* to human cervical epithelia (tCX) and human urethral epithelial (tUEC) cells with *N. gonorrhoeae* 1291 WT, Δ NHBA, and Δ NHBA_C strains. *C*, Adherence of *N. gonorrhoeae* 1291 WT with tCX cells that were either untreated (no treatment) or pretreated with recombinant *N. gonorrhoeae* NHBA (NHBA_{Ng}) (1–100 μ g/mL) or peanut agglutinin (PNA) as a negative control (100 μ g/mL). Data represent the average percent adherence for triplicate samples as a percentage of that for the inoculum and are shown relative to the WT; the results for the WT, set at 100%, are 1.1×10^5 (tCX) and 1.7×10^5 (tUEC) (*B*) and 6.5×10^4 adherent CFUs (*C*). Error bars represent standard deviations. Experiments were performed ≥ 3 times, and representative results are shown. * $P < .05$; $\dagger P < .01$; $\ddagger P < .001$ (relative to untreated WT strain; 2-tailed Student *t* test).

in the Arg region, known to be involved in NHBA_{Nm} binding to GAGs [11, 12].

Furthermore, *N. gonorrhoeae* NHBA_{Ng} but not NHBA_{Nm} interacts with Lewis X and sialyl Lewis X antigens, LNNt and its sialylated variants on the glycan array, which can typically be found on the surface of host cells. Meningococcal cleavage of NHBA_{Nm} by NalP releases a C-terminal fragment, C2 [11], which contains the Arg-rich region and increases vascular permeability [16]. In addition, human proteases lactoferrin and kallikrein cleave NHBA_{Nm} downstream of the Arg region [11, 14], and the serum C3 convertase can cleave released NHBA C2 fragment, removing the Arg region and negating the toxic effect of protein on cells [15]. Because *N. gonorrhoeae* does not have a *nalP* gene [19], NHBA_{Ng} would not be cleaved upstream of the Arg region. NHBA_{Ng} is not cleaved by human lactoferrin (data not shown), and cleavage by kallikrein and C3 convertase have not been investigated. However, owing to the absence of NalP, even if NHBA_{Ng} is cleaved by these human enzymes, the functional Arg region would remain attached to the gonococcal surface where it can mediate its roles associated with glycan binding.

The gonococcal Δ NHBA mutant strain displayed several phenotypes relative to the WT and Δ NHBA_C strains, including decreased survival in human serum, decreased aggregation and microcolony formation, and decreased adherence to tCX and tUEC cells. Although *N. gonorrhoeae* rarely causes disseminated disease, its ability to resist serum killing has been extensively studied and found to be mediated by factors including porin [27–29], LOS [30], and Opa protein [31], and that ability is relevant during mucosal infections, as serum and complement factors are present in the genital tract and other mucosal surfaces [32–34]. NHBA_{Nm} is also involved in serum survival, with addition of heparin before the serum assay resulting in increased survival of the serum-sensitive, unencapsulated parent meningococcal strain but not the Δ NHBA mutant strain [11].

Heparin interacts with several complement factors [35], and heparin-mediated recruitment of complement regulatory proteins by NHBA_{Nm} was proposed to be the mechanism of action for serum resistance [11]. We propose a similar mechanism of action for NHBA_{Ng}, involving a higher level of recruitment of complement regulatory proteins to the surface of the WT strain relative to the Δ NHBA mutant. Even though NHBA_{Ng} has higher affinity for heparin than NHBA_{Nm}, additional gonococcal heparin-binding proteins play a role in serum resistance, such as Opa protein [31], which would account for the relatively high level of survival of the WT under the serum conditions tested, as well as the restoration of resistance of the gonococcal Δ NHBA mutant after addition of heparin to the assay.

Gonococcal adherence to the mucosal epithelium is the key first step in establishing an infection, and after initial adherence, *N. gonorrhoeae* colonization depends on the formation

of robust bacterial aggregates and microcolonies on the epithelial cell surface (reviewed in [26]). Both initial adherence and microcolony formation are mediated by gonococcal factors, including type IV pili, Opa proteins, and LOS [36–39]. However, *N. gonorrhoeae* also form aggregates even in the absence of pili or Opa protein, suggesting the presence of unknown host factors that facilitate gonococcal aggregation [40].

We show that NHBA_{Ng} plays a key role in establishing gonococcal infection, because the ΔNHBA mutant displayed decreased adherence to tCX and tUEC cells, as well as decreased aggregation and microcolony formation relative to the WT strain. Furthermore, recombinant NHBA_{Ng} directly interacts with both epithelial cells and gonococcal cells, and NHBA_{Ng} can block adherence to epithelial cells in a concentration-dependent manner. This is likely a result of NHBA interactions with host glycans on the epithelial cells, which mediate NHBA_{Nm} interactions with Hec-1B and CHO-K1 cells [12]. The gonococcal NHBA is up-regulated at 32°C versus 37°C, consistent with NHBA regulation in *N. meningitidis* [17], which may be particularly relevant during adherence in the pharynx by these organisms, owing to the lower temperature of this niche.

The NHBA_{Ng}-mediated interbacterium interactions, and the role of NHBA_{Ng} in microcolony formation, may be facilitated by its interactions with LNNt, which is present on the surface of gonococcal cells as part of LOS [41]. Gonococcal microcolonies interact with host microvilli and lead to rearrangement of the host cytoskeleton and cortical plaque formation [42–46]. Microcolonies have also been implicated in increasing gonococcal resistance to antibiotics [40], and formation of bacterial aggregates is enhanced after exposure to seminal plasma, which influences bacterial transmission [47]. The formation of *N. meningitidis* aggregates is also important for resisting shear forces on the cell surface [48], which may also be important for *N. gonorrhoeae*. However, it is interesting to note that meningococcal NHBA has not been reported to be involved in aggregation to date [11, 12, 20].

In summary, we highlight the role of NHBA during several stages of gonococcal infection and pathogenesis. Our findings suggest that targeting NHBA-self and NHBA-host interactions may be a useful therapeutic and vaccine approach.

Notes

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