



Resistance of *Neisseria gonorrhoeae* to neutrophils

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Infection with the human-specific bacterial pathogen *Neisseria gonorrhoeae* triggers a potent, local inflammatory response driven by polymorphonuclear leukocytes (neutrophils or PMNs). PMNs are terminally differentiated phagocytic cells that are a vital component of the host innate immune response and are the first responders to bacterial and fungal infections. PMNs possess a diverse arsenal of components to combat microorganisms, including the production of reactive oxygen species and release of degradative enzymes and antimicrobial peptides. Despite numerous PMNs at the site of gonococcal infection, *N. gonorrhoeae* can be cultured from the PMN-rich exudates of individuals with acute gonorrhea, indicating that some bacteria resist killing by neutrophils. The contribution of PMNs to gonorrheal pathogenesis has been modeled *in vivo* by human male urethral challenge and murine female genital inoculation and *in vitro* using isolated primary PMNs or PMN-derived cell lines. These systems reveal that some gonococci survive and replicate within PMNs and suggest that gonococci defend themselves against PMNs in two ways: they express virulence factors that defend against PMNs' oxidative and non-oxidative antimicrobial components, and they modulate the ability of PMNs to phagocytose gonococci and to release antimicrobial components. In this review, we will highlight the varied and complementary approaches used by *N. gonorrhoeae* to resist clearance by human PMNs, with an emphasis on gonococcal gene products that modulate bacterial-PMN interactions. Understanding how some gonococci survive exposure to PMNs will help guide future initiatives for combating gonorrheal disease.

Keywords: *Neisseria gonorrhoeae*, virulence factors, neutrophils, polymorphonuclear leukocytes, phagocytosis, reactive oxygen species, antimicrobial peptides, neutrophil proteases

GONORRHEAL DISEASE

Gonorrhea is a major global health problem, with greater than 62 million cases estimated to occur worldwide per year (Anonymous, 2001). Numbers of reported cases in the United States have remained at approximately 330,000 annually, but it is estimated that the actual number is at least twice as high, and rates of gonorrhea are rapidly increasing among men who have sex with men and young adults (Workowski and Berman, 2010). The cause of gonorrhea is the Gram-negative diplococcus *Neisseria gonorrhoeae* (the gonococcus or Gc). Gc is a human-specific pathogen that is transmitted via close sexual contact with an infected individual. Gonorrhea presents as an acute urethritis in men and cervicitis in women, but the pharynx and rectum can also be infected (Wiesner and Thompson, 1980). Because of the frequently asymptomatic nature of female infection, gonorrhea is a major cause of pelvic inflammatory disease, characterized by abdominal pain and tubal scarring that results in ectopic pregnancy and infertility; untreated infections in men also lead to sterility. Disseminated Gc infections can cause arthritis–dermatitis syndrome, endocarditis, and meningitis. Gc can also be vertically transmitted during childbirth and is still a leading cause of infectious neonatal blindness in the developing world (Wiesner and Thompson, 1980). Gc remains a major public health problem due to rapid acquisition of resistance to multiple antibiotics (Tapsall, 2009) and its ability to phase and antigenically vary its surface structures, preventing infected individuals from developing a protective immune response and hindering development of a protective vaccine (Virji, 2009).

Regardless of the anatomic site that is infected, Gc promotes an inflammatory response that is characterized by the recruitment of PMNs (Figure 1). In men, PMNs appear in urethral swabs and urine several days after infection and immediately prior to the onset of symptoms (Cohen and Cannon, 1999). The purulent exudate produced by infected men, described in the Bible and by Galen, is the best-known aspect of gonorrheal disease and is reflected in the translation of “gonorrhea” from Greek as “flow of seed” (Edwards and Apicella, 2004). The cervical secretions of women with gonorrhea also contain PMNs (Evans, 1977). Bacteria in gonorrheal secretions are attached to and within PMNs (Ovcinnikov and Delektorskij, 1971; Farzadegan and Roth, 1975; Evans, 1977; King et al., 1978; Apicella et al., 1996). PMNs are the primary innate immune responders to bacterial and fungal infection and are capable of phagocytosing and killing a variety of microorganisms (Borregaard, 2010). Yet in spite of the numerous PMNs at the site of gonorrheal infection, viable Gc can be cultured from the exudates of infected individuals (Wiesner and Thompson, 1980), and a subset of Gc remain viable when Gc are exposed to PMNs *in vitro* (see below). We interpret these results to show that the PMN-driven innate immune response to Gc is ineffective at clearing a gonorrheal infection. The persistence of Gc in the presence of PMNs facilitates Gc's long-term colonization of its obligate human hosts, creating enhanced opportunity for dissemination and transmission of gonorrhea. In this review we will highlight our current knowledge about Gc resistance to PMN clearance, a critical aspect of the virulence of Gc.

PMN ANTIMICROBIAL ACTIVITIES

PMNs are the most abundant white cells in the peripheral blood of humans. They are professional phagocytes and the first line of defense of the innate immune system (Borregaard, 2010). In response to peripheral infection or damage, PMNs follow chemotactic cues to extravasate from the bloodstream and migrate through tissues to reach the target site. Mucosal epithelial cells and resident immune release chemokines for PMNs, including interleukin-8, interleukin-6, tumor necrosis factor- α , and interleukin-1 (Borregaard, 2010). These chemokines are released during human Gc infection (Ramsey et al., 1995; Hedges et al., 1998).

PMNs possess receptors to bind and phagocytose complement- and antibody-opsonized particles [e.g., complement receptor 3 (CR3), FcRs]. They can also engulf unopsonized particles through lectin-like interactions or using receptors that are specific for ligands on the particle surface (Groves et al., 2008). Interaction between PMNs and a target particle results in the mobilization of different subsets of cytoplasmic granules to the plasma or phagosomal membrane (Figure 2). Granule fusion enables the degradation and killing of microorganisms both intracellularly and extracellularly (Borregaard et al., 2007). PMN mechanisms of microbial killing include production of reactive oxygen species (ROS) via the NADPH oxidase enzyme (the “oxidative burst”) as well as the oxygen-independent activities of degradative enzymes and antimicrobial peptides (Table 1). Human PMN granules are classified as azurophilic or primary granules, which contain myeloperoxidase, α -defensin peptides, and cathepsin G, among other antimicrobial components; specific or secondary granules containing the flavocytochrome b_{558} subunit of NADPH oxidase, LL-37 cathelicidin, lactoferrin, and CR3; and gelatinase or tertiary granules containing gelatinase (Borregaard et al., 2007). PMN granules release their contents in a set order. Initially, gelatinase granule contents degrade extracellular matrix, allowing PMNs to migrate across the tissues underlying the site of infection. Next, the release of specific granules at the target destination increases phagocytic potential due to presentation of CR3 on the PMN surface.

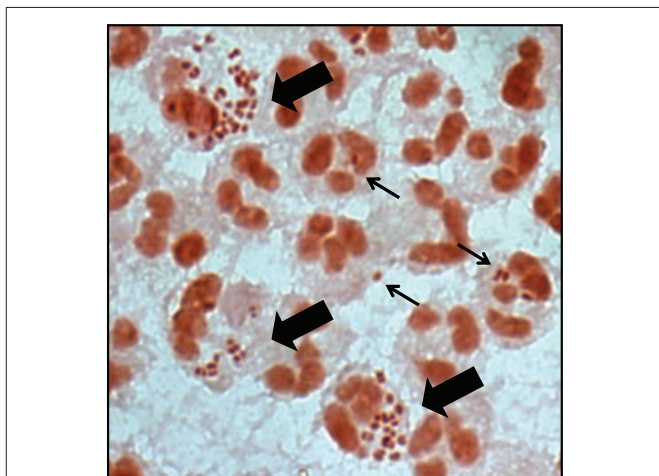


FIGURE 1 | Gonorrhoeal exudates contain numerous PMNs with associated Gc. Gram stain of the urethral exudate from a male with uncomplicated gonorrhea. Some PMNs associate with single diplococci (thin arrow), while others have multiple adherent and internalized Gc (thick arrow). Note that the majority of PMNs in the exudate are uninfected.

Finally, the release of both specific and azurophilic granules creates an environment that is generally hostile to microbial survival (Lacy and Eitzen, 2008). PMNs also release neutrophil extracellular traps (NETs) composed of DNA, histones, and selected granule components, which trap and kill microbes without requiring phagocytosis (Papayannopoulos and Zychlinsky, 2009). Thus PMNs combine oxygen-dependent and -independent mechanisms to combat intracellular and extracellular microorganisms.

The fact that gonorrhoeal exudates contain viable Gc indicates that PMNs are ineffective at completely clearing Gc infection. There are two mechanisms that could explain how Gc survives PMN challenge: Gc prevents PMNs from performing their normal antimicrobial functions (phagocytosis, granule content release), or Gc expresses defenses against oxidative and non-oxidative components produced by PMNs (Figure 2). As we will discuss, there is now substantial evidence for both mechanisms, which ultimately enable Gc to survive within a host and be transmitted to new individuals.

MODEL SYSTEMS FOR EXAMINING PMNS DURING Gc PATHOGENESIS

Four experimental approaches have been used to investigate the involvement of PMNs in gonorrhoeal disease. Each has contributed to our understanding of how PMNs are recruited during acute gonorrhea and how Gc withstands this onslaught.

THE MALE URETHRAL CHALLENGE MODEL

Experimental human infection is limited to male urethral inoculation, due to the potential for severe complications such as pelvic inflammatory disease in women with gonorrhea (Cohen and

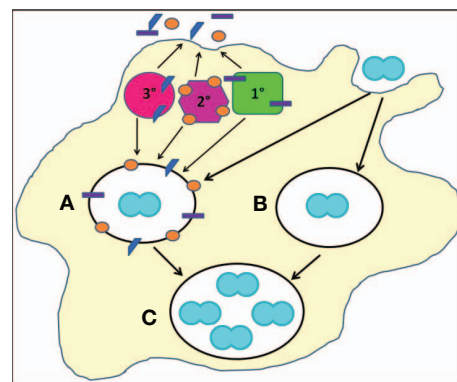


FIGURE 2 | Cellular mechanisms of Gc survival after exposure to PMNs.

Gc (blue diplococcus) attaches to the surface of PMNs and is engulfed into a phagosome (white oval). PMNs possess three classes of granules (1°, 2°, and 3°), each of which contains a unique subset of antimicrobial compounds. Granules fuse with the nascent phagosome or plasma membrane to deliver their contents to invading microorganisms. We propose two mechanisms that allow Gc to survive after exposure to PMNs. (For illustrative purposes, only intracellular Gc survival is depicted.) First, PMN granules release their contents at the plasma membrane or into phagosomes containing Gc (A). However, Gc virulence factors confer resistance to granules' antimicrobial compounds. Second, Gc prevents PMN granules from releasing their contents at the plasma membrane or into phagosomes, allowing the bacteria to avoid encountering PMN antimicrobial compounds (B). Either mechanism would enable a fraction of Gc to survive and replicate in the presence of PMNs (C).

Table 1 | Antimicrobial components housed in PMN granules.

Granule class	Granule components
Primary/azurophilic	<i>Cathepsin G</i> , <i>BPI</i> , <i>lysozyme</i> , elastase, myeloperoxidase (MPO), α -defensins
Secondary/specific	<i>Flavocytochrome b₅₅₈</i> , <i>LL-37 (hCAP18)</i> , <i>lysozyme</i> , gelatinase, lactoferrin, CD11b/CD18 (CR3)
Tertiary/gelatinase	<i>Flavocytochrome b₅₅₈</i> , <i>lysozyme</i> , gelatinase, CD11b/CD18 (CR3)

Proteins that have been shown to have or produce antimicrobial activity against Gc *in vitro* are bolded and italicized. Proteins to which Gc is resistant are indicated in red type.

Cannon, 1999). Urethral infection of male volunteers results in the release of proinflammatory cytokines and appearance of PMNs in the urogenital tract 2–3 days after infection, similar to what is seen in natural cases of gonococcal urethritis (Cohen and Cannon, 1999). As in natural infections, exudates from males with experimental Gc infection contain PMNs with associated Gc and occasional exfoliated epithelial cells. Electron microscopic analysis of these exudates revealed that a subset of Gc inside PMNs appear intact, providing the initial evidence that Gc may survive within PMN phagosomes (Ovcinnikov and Delektorskij, 1971; Farzadegan and Roth, 1975; Apicella et al., 1996).

THE FEMALE MURINE GENITAL TRACT MODEL

Dr. Ann Jerse (Uniformed Services University of the Health Sciences) has developed a female mouse model of Gc genital tract infection, which allows gonorrheal infection to be examined in a genetically tractable host. In this model, estradiol-treated mice are inoculated vaginally with Gc, which allows over 80% of mice to be colonized with bacteria for over 1 week. Infected mice produce inflammatory cytokines, leading to rapid appearance of PMNs in the genital tract (Jerse, 1999). Experimental infection of female mice has provided insight into the selective advantage of opacity-associated (Opa) protein expression on Gc survival and the roles of Gc virulence factors conferring *in vitro* resistance to ROS and antimicrobial peptides in *in vivo* infection (Jerse, 1999; Jerse et al., 2003; Wu and Jerse, 2006; Wu et al., 2009; Cole et al., 2010). Because mice lack the human-specific receptors and other components that are likely to be important for gonorrheal disease, future studies could employ mice transgenic for human proteins of interest. Inbred mice that are transgenic for human carcinoembryonic antigen-related cellular adhesion molecules (CEACAMs) and CD46, receptors that are implicated in gonorrheal pathogenesis (Merz and So, 2000), have already been developed (Johansson et al., 2003; Gu et al., 2010), with additional mouse strains likely to be produced in the coming years.

IMMORTALIZED PMN-LIKE CELL LINES

The use of immortalized promyelocytic human cell lines to study the molecular mechanisms of Gc pathogenesis provides a system which is clonal, easy to maintain, and amenable to expression of transgenes. As one example, the leukemic HL-60 cell line can be differentiated into a PMN-like phenotype with retinoic acid (Collins et al., 1977; Newburger et al., 1979). Differentiated HL-60 cells can

phagocytose and generate ROS in response to Opa-expressing Gc akin to primary human PMNs (Bauer et al., 1999; Pantelic et al., 2004). However, HL-60 cells do not possess the robust antimicrobial activity associated with primary cells, due in part to the absence of specific granules and other intracellular compartments (Le Cabec et al., 1997).

PRIMARY PMNs

Research on the molecular mechanisms underlying Gc infection of PMNs has mostly relied upon primary human cells, purified from freshly isolated human blood. The abundance of PMNs in human blood and the ease of purification make PMNs amenable to infection with Gc *in vitro*. The limitations of working with primary PMNs include their short half-life, their limited capacity for genetic manipulation, and the person-to-person variability intrinsic to primary human cells. However, primary human PMNs have been used to measure binding and phagocytosis of Gc, quantify Gc survival after PMN exposure, and assess the roles of Gc virulence factors in bacterial defense against PMNs (see below). Gc infection of murine PMNs has also been conducted (Wu and Jerse, 2006; Soler-Garcia and Jerse, 2007). Future studies using primary PMNs along with cultured epithelial cells from relevant anatomic sites may provide a means to examine the complex interactions between host cells that occur during gonorrheal infection.

Gc interaction with PMNs is influenced by the physiological state of the PMNs being used. Initial experimentation with primary human PMNs utilized cells and Gc suspended in buffered saline solutions (Densen and Mandell, 1978; Rest et al., 1982), but this is unlikely to reflect the transmigrated, primed state of PMNs in the genitourinary tract during acute infection. Research from the laboratory of Dr. Richard Rest (Drexel University) demonstrated that when PMNs were allowed to adhere to tissue culture-treated dishes, they released granule components and bound significantly more Gc than PMNs in suspension (Farrell and Rest, 1990). Dr. Michael Apicella's laboratory (University of Iowa) subsequently developed an assay using collagen-adherent PMNs, which generated a system for studying the role of selected Gc virulence factors in bacterial survival after PMN challenge (Seib et al., 2005; Simons et al., 2005). We adapted the Apicella protocol to include PMN treatment with the chemokine interleukin-8, which facilitates PMN activation (Borregaard, 2010). We used this system to demonstrate Gc survival inside PMNs and to identify Gc proteins that defend the bacteria from killing by PMNs (Stohl et al., 2005; Criss et al., 2009).

Gc SURVIVAL AND REPLICATION IN THE PRESENCE OF PMNs

Although the survival of Gc in association with PMNs was once hotly debated, there is now substantial evidence that gonococci survive and multiply within human phagocytes. Examination of urethral exudates by light and electron microscopy has repeatedly shown the presence of abundant PMNs with associated and internalized Gc (Ovcinnikov and Delektorskij, 1971; Farzadegan and Roth, 1975; King et al., 1978; Apicella et al., 1996). The fact that viable gonococci can be cultured from urethral exudates or cervical swabs is strongly suggestive of Gc survival in the presence of PMNs (Wiesner and Thompson, 1980). *In vitro* studies from the Apicella laboratory using adherent human PMNs demonstrated that over 50% of Gc internalized by PMNs remained viable for

up to 6 h, as determined by viable bacterial counts and electron microscopy (Simons et al., 2005). Our group corroborated these findings and directly detected viable extracellular and intracellular Gc after PMN infection, using dyes that reveal the integrity of bacterial membranes (Criss et al., 2009). We conclude from these studies that a fraction of Gc can survive both extracellularly and intracellularly in the presence of PMNs.

There is evidence that Gc does not only persist within PMNs, but also uses the PMNs as a site for replication. Pioneering studies in the 1970s showed that Gc inside exudate-derived PMNs were sensitive to penicillin, which only kills replicating bacteria. In the presence of antimicrobial agents such as spectinomycin or pyocin that cannot permeate eukaryotic membranes, numbers of PMN-associated Gc increased over time, indicative of bacterial replication inside exudatous and *in vitro*-infected PMNs (Veale et al., 1976, 1979; Casey et al., 1979, 1980, 1986). Using electron microscopy and colony counts, the Apicella laboratory observed an increase in Gc within collagen-adherent human PMNs over a 6-h infection, results also suggestive of intracellular replication (Simons et al., 2005). Similarly, we used bacterial viability dyes to observe an increase in the number of viable Gc inside PMNs over time (Criss et al., 2009). While the advantage of Gc replicating inside terminally differentiated cells of a limited life span is questionable, the Apicella group showed that PMNs infected with Gc delay their spontaneous apoptosis (Simons et al., 2006). We anticipate that advances in cellular imaging will provide additional support for Gc replication inside PMNs and will give insight into the timing and extent of this event.

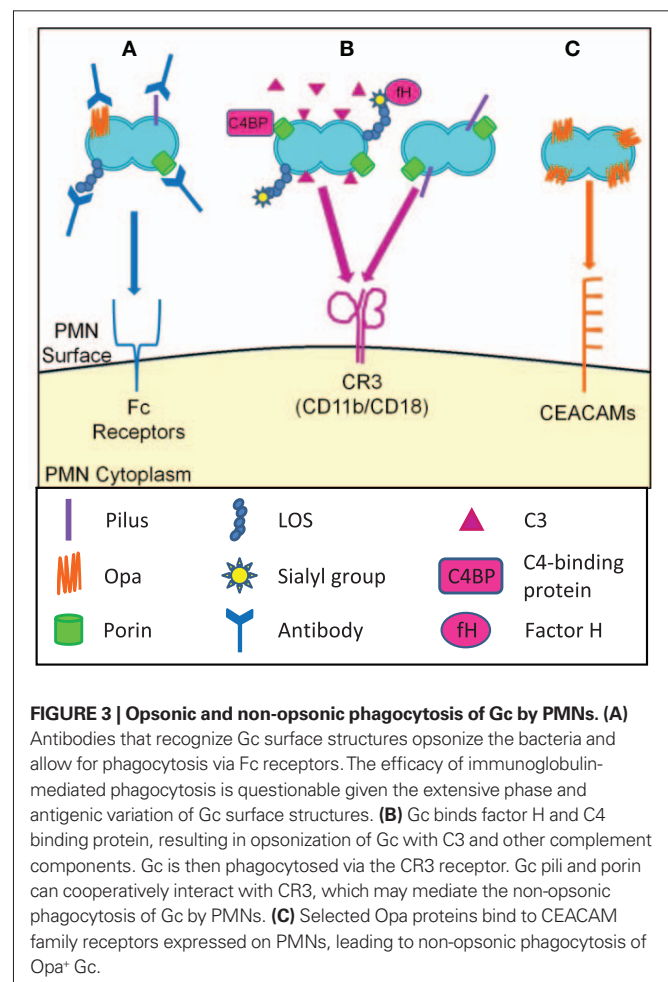
BINDING AND PHAGOCYTOSIS OF Gc BY PMNs

Since gonorrheal secretions contain PMNs associated with viable intracellular and extracellular bacteria, Gc must possess factors that promote attachment and phagocytosis by PMNs. Opsonic and non-opsonic interactions are the two basic means of phagocytosis, both of which may be utilized by Gc (Groves et al., 2008; Figure 3).

OPSONIC UPTAKE

The two major opsonins for PMN phagocytosis are immunoglobulins and complement, which bind to Fc receptors and complement receptors such as CR3, respectively (Groves et al., 2008). Patients with gonorrhea produce opsonic IgG and IgA directed against Gc surface-exposed components including porin, Opa proteins, pilin, iron-regulated outer membrane proteins, and lipooligosaccharide (LOS) (Brooks et al., 1976; McMillan et al., 1979; Tramont et al., 1980; Rice and Kasper, 1982; Siegel et al., 1982; Lammel et al., 1985; Schwalbe et al., 1985). Intriguingly, serum from individuals with no prior history of gonorrhea contains opsonic IgG against Gc porin and IgM against Gc LOS isotypes containing hexosamine; the non-Gc antigens recognized by these antibodies are not known (Sarafian et al., 1983; Griffiss et al., 1991). Many of the Gc surface structures that promote humoral immune responses are phase and antigenically variable and thus evade antibody-mediated immune surveillance (Virji, 2009). Also, Gc secretes an IgA protease that cleaves the polymeric IgA in mucosal secretions (Blake and Swanson, 1978). Thus complement rather than antibodies is likely to drive the opsonic phagocytosis of Gc by PMNs.

The complement system is a key component of the innate immune system comprised of more than 30 proteins. The complement system can be activated by three routes: the classical, the alternative, and the lectin pathway, but all three routes normally proceed to proteolytic activation of the major complement protein C3 and assembly of the membrane attack complex (Ram et al., 2010). Gc has multiple ways of resisting the bactericidal activities of complement in normal human serum. Gc binds the complement regulatory proteins C4b-binding protein (C4BP) and factor H (fH) on its surface via porins and sialylated LOS (Ram et al., 1998a,b, 2001; Gulati et al., 2005). C4BP restricts the amount of C3 which can be deposited by the classical complement pathway. fH is a cofactor for factor I-mediated cleavage of C3b to the hemolytically inactive iC3b. In the alternative pathway fH irreversibly dissociates factor Bb to limit C3 deposition and subsequent C5 cleavage (Ram et al., 2010). C4BP and fH provide defense against direct complement-mediated killing but concomitantly increase iC3b deposition on the Gc surface. iC3b is a ligand for CR3 (CD11b/CD18), which in PMNs drives actin-dependent particle engulfment into degradative phagolysosomes and production of ROS (Groves et al., 2008). Although it is assumed that Gc is complement-opsonized at mucosal surfaces, how opsonization impacts Gc survival after PMN exposure remains to be explored.



NON-OPSONIC UPTAKE

In the absence of antibodies or complement, efficient binding and engulfment of Gc by PMNs is achieved via expression of colony Opa proteins (King and Swanson, 1978; Virji and Heckels, 1986; Fischer and Rest, 1988). Opa proteins, formerly known as “protein II,” are a family of closely related, 20–30 kD outer membrane proteins that facilitate Gc binding and internalization by human cells, including PMNs (Sadarangani et al., 2011). Gc strains possess approximately 11 *opa* genes encoding 7–8 antigenically distinct Opa proteins (Connell et al., 1990; Dempsey et al., 1991). Each *opa* gene is phase-variable due to slipped-strand mispairing in a pentameric nucleotide repeat that places the gene in or out of frame (Murphy et al., 1989), such that individual Gc can express zero, one, or any possible combination of Opa proteins. Differential expression of Opa proteins can influence bacterial tropism for host cell types and provides a mechanism of immune evasion (Sadarangani et al., 2011).

Opacity-associated proteins bind heparan sulfate proteoglycans (HSPGs) and/or CEACAMs. Only those Opa proteins that bind CEACAMs are reported to influence Gc interactions with PMNs (Sadarangani et al., 2011). The Opa-binding CEACAMs on PMNs are CEACAM1, CEACAM3, and CEACAM6, with CEACAM3 expression exclusively restricted to PMNs. CEACAM1 and CEACAM3 are transmembrane proteins, while CEACAM6 possesses a glycosylphosphatidylinositol anchor (Gray-Owen and Blumberg, 2006). Binding of Opa proteins to any of the three CEACAMs results in Gc internalization, but via different signaling events (McCaw et al., 2004).

Opacity-associated protein expression is selected for in the male urethra, the female cervix during the follicular phase of the menstrual cycle, and in the murine cervix (James and Swanson, 1978; Swanson et al., 1988; Jerse et al., 1994; Jerse, 1999). However, Opa⁺ Gc survives better after exposure to PMNs *in vitro* than isogenic Opa⁺ Gc (Rest et al., 1982; Virji and Heckels, 1986; Criss et al., 2009). Opa protein expression increases Gc phagocytosis by PMNs and stimulates PMN ROS production, and both factors may influence bacterial survival after exposure to PMNs (Rest et al., 1982; Fischer and Rest, 1988).

Gc surface structures other than Opa proteins may contribute to adherence and phagocytosis by PMNs. Pili and porin cooperatively interact with CR3 on cervical epithelial cells (Edwards et al., 2002). It is not known if this interaction occurs on PMNs, but if it were to occur, it would drive non-opsonic uptake of Gc by PMNs. *In vitro* studies suggested that “type 1,” virulent, piliated Gc were resistant to phagocytosis and killing by PMNs compared to “type 4,” avirulent, non-piliated bacteria that arise after extensive laboratory passage (Ofek et al., 1974; Dilworth et al., 1975). We now know that type 1 and type 4 Gc vary in Opa expression as well as piliation, both of which could have contributed to these observations. Purified porins also decrease PMN actin polymerization, which may reduce the phagocytosis of Gc by PMNs (Bjerknes et al., 1995). Serogroup C strains of *N. meningitidis* with lacto-N-neotetraose (LNnT) on LOS are phagocytosed by neutrophils in an opsonin-independent manner (Estabrook et al., 1998); it has not been examined whether this LOS epitope on Gc affects phagocytosis by PMNs. Together, the combinatorial expression of Opa proteins, pili, porin, and LOS modulate Gc binding and internalization by PMNs.

Gc DEFENSES AGAINST PMN ANTIMICROBIAL ACTIVITIES

Whether they remain extracellular or are phagocytosed by PMNs, Gc must contend with the variety of oxidative and non-oxidative antimicrobial components produced by PMNs (Figure 2). Gc isolated directly from human material or guinea pig subcutaneous chamber fluid display increased survival in the presence of phagocytes compared to Gc grown *in vitro* (Witt et al., 1976; Veale et al., 1977), suggesting that Gc possesses factors necessary for defending against phagocyte killing that are lost or altered with extended *in vitro* culture. These Gc factors aid Gc in resisting the toxic activities of PMNs in two ways. First, Gc prevents PMNs from producing or releasing antimicrobial components. Second, Gc expresses virulence factors that defend against these components. As we will describe, many Gc gene products have been identified that protect Gc from purified ROS, proteases, or antimicrobial peptides, but in most cases their roles in defense against PMNs have not yet been investigated.

DEFENSES AGAINST OXIDATIVE DAMAGE

The major species of ROS include superoxide anion, hydrogen peroxide, and hydroxyl radical. These ROS have different reactivities and half-lives, but together they induce DNA, protein, and cell membrane damage that can lead to cell death (Fang, 2004). There are at least four potential sources of oxidative stress for Gc *in vivo*. (1) PMN NADPH oxidase transports electrons across the phagosomal or plasma membrane to generate superoxide, which spontaneously dismutates to hydrogen peroxide. In PMNs, the azurophilic enzyme myeloperoxidase uses hydrogen peroxide as a substrate to generate hypochlorous acid (bleach; Roos et al., 2003). Phagocytes can also produce reactive nitrogen species (RNS) such as nitric oxide and peroxynitrite, but RNS appear to be of limited importance in human PMN antimicrobial activity (Fang, 2004). (2) Enzymes related to phagocyte NADPH oxidase are expressed in epithelial cells, and the survival defect of Gc antioxidant mutants inside primary cervical cells implies that epithelial cells may also be an important source of oxidative stress for Gc (Wu et al., 2005, 2006; Achard et al., 2009; Potter et al., 2009). (3) *Lactobacillus* species that generate hydrogen peroxide are normally found in the vaginal flora of women (Eschenbach et al., 1989). Women with inhibitory lactobacilli are less likely to be infected with Gc (Saigh et al., 1978), and lactobacilli inhibit Gc growth *in vitro* (Saigh et al., 1978; Zheng et al., 1994; St Amant et al., 2002). However, it appears that effects of lactobacilli on Gc may be independent of hydrogen peroxide production, since mucosal secretions can effectively quench lactobacilli-derived ROS (O’Hanlon et al., 2010). (4) Gc generate ROS during aerobic respiration, although this may be less of an issue *in vivo*, where the oxygen tension in the genitourinary tract is low (Archibald and Duong, 1986). Gc defenses against oxidative stress involve manipulation of the PMN oxidative burst, detoxifying or repair of oxidative damage, and transcriptional upregulation of antioxidant gene products (Figure 4A).

Gc manipulation of the PMN oxidative burst

In the absence of Opa protein expression, Gc fails to induce the PMN oxidative burst (Rest et al., 1982; Virji and Heckels, 1986; Fischer and Rest, 1988; Criss and Seifert, 2008). Even in the presence of Opa⁺ Gc that induce ROS production in PMNs, the magnitude of ROS production is small relative to stimuli such as phorbol esters or other

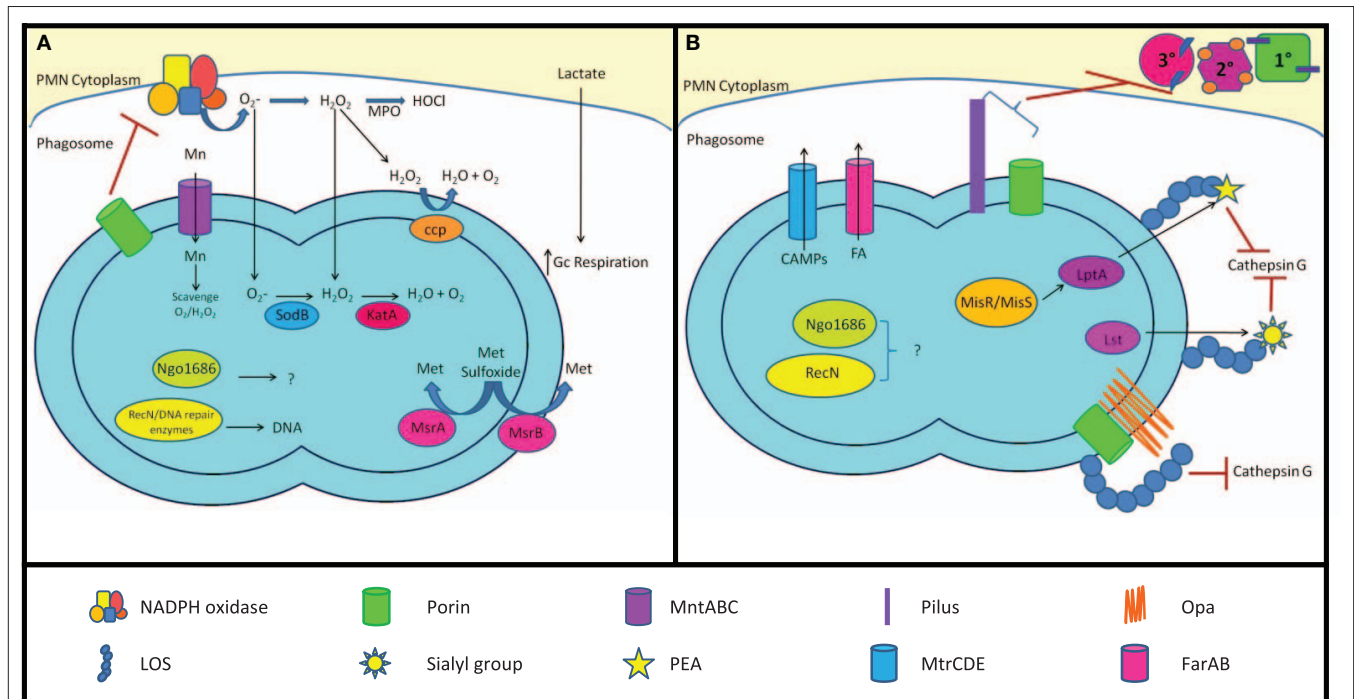


FIGURE 4 | Mechanisms of Gc survival after exposure to antimicrobial compounds produced by PMNs. (A) Resistance to oxidative damage. PMN NADPH oxidase generates superoxide (O_2^-) and hydrogen peroxide (H_2O_2) from O_2 , which are converted to hypochlorous acid (HOCl) by myeloperoxidase (MPO). Gc prevents PMNs from generating ROS by lactate-mediated increase in Gc O_2 consumption and suppression of NADPH oxidase activity by porin or as-yet unidentified factors. Gc scavenges ROS through the activities of MntABC, superoxide dismutase (SodB), catalase (KatA), and cytochrome c peroxidase (Ccp). Gc can also repair damage due to ROS through DNA repair enzymes (RecN), protein reductases (MsrA/B), and other proteins (Ngo1686). **(B)** Resistance to PMN non-oxidative damage. Gc pili and/or porins prevent PMN

granules from releasing non-oxidative antimicrobial components. LOS protects Gc outer membrane proteins such as Opa and porin from proteolysis by cathepsin G. Sialylation of LOS by Lst and PEA modification of LOS by LptA increases bacterial resistance to cathepsin G and other antimicrobials. The MisR/MisS two-component regulator increases expression of LptA and other gene products that confer resistance to PMN non-oxidative damage. Ngo1686 and RecN also protect Gc from PMN non-oxidative damage. The MtrCDE and FarAB efflux pumps export cationic antimicrobial peptides (CAMPs) and long-chain fatty acids (FA) from the Gc cytoplasm, respectively. In most cases, the contribution of these virulence factors to Gc survival after exposure to PMNs remains to be determined.

bacteria (Simons et al., 2005). Gc utilizes three mechanisms to reduce the amount of ROS produced by PMNs. First, exposure to lactate that is released from PMNs undergoing glycolysis stimulates the rate of Gc oxygen consumption, reducing the amount available to PMNs as a substrate for NADPH oxidase (Britigan et al., 1988). Second, purified Gc porin inhibits PMN ROS production in response to Gc, yeast particles, and latex beads (Lorenzen et al., 2000), but not formylated peptides (Haines et al., 1988; Bjerknes et al., 1995). Whether porin has this effect in the context of whole Gc bacteria remains to be examined. Third, we reported that Opa⁻ Gc suppresses the PMN oxidative burst induced by serum opsonized staphylococci and formylated peptides by a process requiring bacterial protein synthesis and bacteria-PMN contact; the bacterial products mediating this effect are not known at this time (Criss and Seifert, 2008).

Detoxification and repair of oxidative damage

Bacteria respond to oxidative stress by catalysis of superoxide to hydrogen peroxide by superoxide dismutase (SOD), which is then converted to water and molecular oxygen by catalases and peroxidases (Seib et al., 2006). Gc possesses a single cytoplasmic superoxide dismutase (SodB), one cytoplasmic catalase (KatA), and several genes annotated as peroxidases. SodB activity is low in Gc and does not

play a significant role in protection against oxidative stress (Tseng et al., 2001). In comparison, KatA is crucial to Gc defense against ROS. Gc has approximately 100-fold higher levels of catalase than *E. coli* (Hassett et al., 1990). Disruption of *katA* significantly reduces Gc survival to hydrogen peroxide and superoxide *in vitro* (Johnson et al., 1993; Soler-Garcia and Jerse, 2004; Stohl et al., 2005) and reduces the survival of some strains of Gc in the female murine genital tract (Wu et al., 2009). Gc also has high peroxidase Gc activity due to the periplasmic cytochrome c peroxidase encoded by *ccp* (Archibald and Duong, 1986). *ccp* mutant Gc show slight sensitivity to hydrogen peroxide, which is markedly enhanced when *katA* is also inactivated (Turner et al., 2003). Gc also imports Mn(II) into its cytoplasm via the MntABC transporter, where it scavenges superoxide and hydrogen peroxide by a mechanism independent of SodB and catalase (Tseng et al., 2001). This system is similar to the manganese transport system in *Lactobacillus plantarum* (Archibald and Duong, 1984).

Gc can also repair oxidative damage to proteins and DNA. Gc expresses two forms of methionine sulfoxide reductase, which reverses the oxidation of methionine residues in proteins. The MsrA protein is localized to the cytoplasm, while MsrB is secreted to the outer membrane. A *msrAB* mutant is more sensitive to hydrogen peroxide and superoxide *in vitro* than its wild-type parent (Skaar et al., 2002).

Many Gc gene products involved in recombinational DNA repair, base excision repair, and nucleotide excision repair participate in Gc defense against ROS, such as the recombinase RecA and the DNA-binding protein RecN (Davidsen et al., 2005; Stohl et al., 2005; Stohl and Seifert, 2006; LeCuyer et al., 2010). The putative metalloprotease Ngo1686 helps protect Gc from hydrogen peroxide and the lipid oxidant cumene hydroperoxide, but the cellular targets with which Ngo1686 interacts are currently unknown (Stohl et al., 2005). Both *ngo1686* and *recN* mutants have significant survival defects after exposure to primary human PMNs, but a *recA* mutant does not (Stohl et al., 2005; Criss et al., 2009).

Transcriptional induction of antioxidant gene products

Gc pre-exposed to hydrogen peroxide survives PMN challenge significantly better than unexposed Gc (Criss et al., 2009). This finding implies that Gc possesses complex transcriptional circuitry that is important for defenses against ROS and/or PMNs. The transcriptome of Gc exposed to sublethal concentrations of hydrogen peroxide was defined and revealed the upregulation of transcripts encoding RecN, Ngo1686, and other antioxidants after oxidative challenge (Stohl et al., 2005). Antioxidant gene expression is regulated by selected transcriptional repressors. The OxyR protein represses KatA expression, which is relieved following oxidative stress in order to increase catalase production (Tseng et al., 2003). PerR is responsive to Mn(II) levels and represses expression of MntC, part of the Mn(II) transporter (Wu et al., 2006). Finally, Ngo1427, a LexA homolog, represses expression of RecN, which is relieved when a cysteine residue is oxidized (Schook et al., 2011).

PMNs PRIMARILY DIRECT NON-OXIDATIVE ANTIMICROBIAL COMPONENTS AGAINST Gc

Although Gc has complex mechanisms for detecting oxidative damage and responding to it, the importance of these processes in Gc survival to PMNs appears to be limited. Mutants in *katA*, *sodB*, *ccp*, or *mntABC*, alone or in combination, do not affect the percentage of Gc that can survive PMN challenge (Seib et al., 2005; Criss et al., 2009). Moreover, Gc survival is similar between normal PMNs and ROS-deficient PMNs obtained from patients with chronic granulomatous disease (CGD; Rest et al., 1982; Criss and Seifert, 2008), and PMNs maintained in anoxic conditions, as are likely to be found in the upper reproductive tract of females, are not impaired for antigenococcal activity (Casey et al., 1986; Frangipane and Rest, 1992). Our group showed that Gc survival was unaffected after exposure to PMNs treated with diphenyleneiodonium (DPI), an inhibitor of NADPH oxidase. DPI treatment or CGD PMNs did not increase the percent survival of *ngo1686* or *recN* Gc, nor did it enhance survival of Opa⁺ Gc that induce ROS from PMNs (Criss et al., 2009). From these results, we conclude that PMNs primarily direct non-oxidative antimicrobial activities against Gc. The functional redundancy in Gc antioxidant defenses may be sufficient to counter PMN-derived ROS; alternatively, PMNs may not generate enough ROS during infection to affect Gc survival.

DEFENSES AGAINST NON-OXIDATIVE DAMAGE

Seminal research from the Rest and Shafer laboratories indicated that components found inside PMN granules display oxygen-independent antigenococcal activity (Rest, 1979; Casey et al., 1985; Rock and Rest, 1988). These components include the bactericidal/

permeability-increasing protein (“hCAP57”), cathepsin G protease, and LL-37 antimicrobial peptide (Casey et al., 1985; Shafer et al., 1986, 1998). Unlike many Gram-negative bacteria, Gc are highly resistant (>0.2 mg/ml) to another class of antimicrobial peptides, the defensins (Qu et al., 1996), although the observed resistance varies depending on experimental conditions used (Porter et al., 2005). Of PMN non-oxidative granule components, cathepsin G and LL-37 have been the most actively studied for their effects on Gc.

Cathepsin G is a highly cationic serine protease which resides in PMN azurophilic granules. It enzymatically cleaves Gc outer membrane proteins including porin and Opa proteins (Rest and Pretzer, 1981; Shafer and Morse, 1987). However, heat and protease inhibitors do not impede cathepsin G’s ability to kill Gc *in vitro*, indicating its antigenococcal activity is independent of its proteolytic activity (Shafer et al., 1986). Cathepsin G can insert into Gc membranes, but killing does not appear to be due to changes in membrane permeability; instead, cathepsin G may impede peptidoglycan biosynthesis (Shafer et al., 1990).

LL-37 is the active form of an 18 kD protein precursor (“hCAP18”) which resides in specific granules. hCAP-18 is proteolytically processed to LL-37 by the azurophilic granule protein proteinase-3 (Sorensen et al., 2001). hCAP-18/LL-37 is also synthesized by mucosal epithelial cells, and is readily detected in cervicovaginal secretions (mean LL-37 concentration of 10 µg/ml) and seminal plasma (mean hCAP-18 concentration of 86 µg/ml; Malm et al., 2000; Tjabringa et al., 2005). Gc infection increases the levels of hCAP18/LL-37 by two- to four-fold in cervical and urethral washes (Porter et al., 2005; Tjabringa et al., 2005). These concentrations of LL-37 would be sufficient to exert antibacterial activity on Gc, since the mean inhibitory concentration of LL-37 for Gc is 6 µg/ml (Shafer et al., 1998). The antigenococcal mechanism of action of LL-37 remains enigmatic and may be related to its ability to form pores that disrupt the integrity of bacterial membranes (Brogden, 2005).

Although Gc are susceptible to cathepsin G and LL-37 *in vitro*, the ability of some percentage of Gc to survive PMN exposure suggests that the bacteria have evolved mechanisms to counter these antimicrobial components. These mechanisms involve direct modulation of PMN granule release, changes to the Gc surface to resist non-oxidative antimicrobial components, and active export of these components (**Figure 4B**).

Modulation of PMN granule release

Both pili and porin have been reported to reduce PMN granule fusion with the plasma membrane or phagosomes. When added to primary PMNs, purified porins inhibit azurophilic and specific granule exocytosis (Bjerknes et al., 1995; Lorenzen et al., 2000). “Type 1,” piliated Gc was also reported to inhibit azurophilic granule exocytosis relative to “type 4,” non-piliated Gc, but additional surface structures expressed on type 1 bacteria may have mediated this result (Densen and Mandell, 1978). More detailed studies with isogenic Gc strains are necessary to determine whether and how Gc surface structures influence granule mobilization.

Modifications to the Gc surface

Gc LOS is thought to mask proteins which are degraded by cathepsin G, since truncation or loss of LOS results in increased binding of cathepsin G and increased susceptibility to cathepsin G-mediated

killing (Shafer, 1988). Two modifications of LOS impact bacterial interactions with host cells and host defenses: phosphoethanolamine (PEA) substitution on lipid A or the oligosaccharide, and sialylation of the terminal Gal β 1-4GlcNAc epitopes of the oligosaccharide (Mandrell et al., 1990; Plested et al., 1999). PEA addition to the heptose group on the beta chain of the core oligosaccharide enhances Gc serum resistance but does not affect susceptibility to antimicrobial peptides (Lewis et al., 2009). In contrast, PEA addition to lipid A by the LptA enzyme increases resistance to both normal human serum and cationic antimicrobial peptides, indicating that structural changes in LOS contribute to the ability of gonococci to resist the bactericidal action of these innate immune components (Lewis et al., 2009). In the related bacterium *N. meningitidis*, expression of *lptA* is positively regulated by the *misR/misS* two-component regulatory system (Newcombe et al., 2005; Tzeng et al., 2008). The roles of MisR/MisS and LptA in Gc pathogenesis remain to be examined. The gonococcal α -2,3-sialyltransferase Lst transfers sialyl groups from host-derived CMP-N-acetylneuraminic acid to the terminal galactose residue on the oligosaccharide of LOS (Gilbert et al., 1996). Sialylation contributes to Gc resistance to normal human serum as well as PMN-derived oxygen-independent antimicrobial factors (Shafer et al., 1986; Parsons et al., 1992). Importantly, sialylated Gc are more resistant to PMNs *in vitro*, and sialylation contributes to Gc survival in the murine female genital tract (Kim et al., 1992; Rest and Frangipane, 1992; Gill et al., 1996; Wu and Jerse, 2006). In addition to LOS, changes in other surface components may contribute to Gc resistance to non-oxidative antimicrobial factors. For instance, loss of Opa expression enhances Gc resistance to serine proteases (Blake et al., 1981; Cole et al., 2010), and *N. meningitidis* lacking pili (due to insertional mutagenesis of the *pilMNOPQ* operon) are more resistant to the model antimicrobial peptide polymyxin B (Tzeng et al., 2005).

Gc export of antimicrobial components

The multiple transferable resistance (*mtr*) locus is a key determinant of Gc resistance to antimicrobial agents (Shafer et al., 1998). Mtr, a member of the resistance-nodulation-cell division (RND) family of efflux pumps, is encoded by a three gene operon designated *mtrCDE*. MtrC spans the periplasm to link the inner membrane protein MtrD, the multidrug efflux transporter, with outer membrane protein MtrE, the channel for export of antimicrobials to the extracellular environment (Hagman et al., 1995). MtrCDE uses the proton motive force to export a variety of compounds from the Gc cytoplasm, including antibiotics, detergents, and antimicrobial peptides (Hagman et al., 1995; Veal et al., 2002). *mtrCDE* is negatively regulated by the MtrR transcriptional repressor (Hagman and Shafer, 1995) and positively regulated by the MtrA transcriptional activator (Rouquette et al., 1999). Mutations in *mtrR* and *mtrA* that modulate expression of MtrCDE affect Gc resistance to antimicrobial peptides (Hagman et al., 1995; Hagman and Shafer, 1995). MtrCDE expression promotes Gc survival in the murine female genital tract (Jerse et al., 2003) and enhances resistance to murine antimicrobial peptides (Warner et al., 2008), but its role in defense of Gc against PMNs is unclear. Gc also uses the FarAB efflux pump system to confer resistance to long-chain fatty acids, independent of Mtr activity (Lee and Shafer, 1999). The Far system is composed of the FarA membrane-spanning linker, the

FarB cytoplasmic membrane transporter, and MtrE. Far expression is believed to be important for survival of isolates at the rectal mucosal surface, which is rich in diet-derived fatty acids, and does not contribute to Gc survival in the murine genital tract (Jerse et al., 2003). How the Far system contributes to defense of Gc against PMNs, which may release fatty acids (Huang et al., 2010), remains to be explored.

DISCUSSION

Despite the prevalence of gonorrhea in the human population and the abundance of PMNs during acute gonorrheal disease, we are just beginning to understand the molecular mechanisms underlying Gc interactions with and resistance to PMNs. There are three overarching questions which remain currently in the field. First, how does a subset of Gc survive PMN challenge? As we have described, Gc possesses gene products which protect against oxidative and non-oxidative components that are made by PMNs. Many of these gene products are necessary for *in vitro* protection against isolated antimicrobial components and some provide a selective advantage *in vivo*. However, in many cases, it has not been investigated whether these gene products also confer a survival advantage in the context of PMN challenge. Second, how does Gc persist over time inside PMNs, as is seen in PMNs isolated from gonorrheal exudates? Although virulence-associated Gc surface structures such as Opa proteins, pili, porin, and LOS have been highly investigated for their biochemistry and impact on Gc-epithelial interactions, their effects on Gc survival inside PMNs remain enigmatic. How complement or immunoglobulin opsonization affects Gc phagocytosis by and survival inside PMNs also needs to be examined. Finally, how and why does Gc stimulate PMN recruitment? That is, what is the benefit of recruiting professional antimicrobial cells to the site of Gc infection? Given the long history of Gc in the human population, Gc could have evolved mechanisms for inhibiting PMN recruitment; instead, Gc LOS and lipoproteins are strong initiators of the host innate immune response (Massari et al., 2002; Pridmore et al., 2003; Zughaier et al., 2004). The answer to this question remains enigmatic, but may be revealed once we have a better understanding of how Gc manipulates PMNs *in vitro* and *in vivo*.

Our current knowledge of Gc interactions with PMNs demonstrates the impressive ability of Gc to survive PMN challenge. Although we are just beginning to piece together the roles of many Gc surface structures and gene products in Gc survival after PMN exposure, we now have model systems in hand that will allow these issues to be directly addressed. We are optimistic that continuing to investigate the mechanisms used by Gc to defend against PMN antimicrobial responses will shed light on how Gc has remained a fixture in the human population for all of recorded history (Wain, 1947; Morton, 1977). This research also has the potential to reveal novel human and Gc targets that can be exploited for new therapeutics to treat the ever-growing threat of highly antibiotic-resistant gonorrhea.

ACKNOWLEDGMENTS

We thank Dr. Jeffrey Tessier for obtaining the clinical sample for the image in **Figure 1**. We thank Louise Ball, Joanna Goldberg, and the two reviewers for helpful comments on the manuscript. This work was supported in part by NIH R00 TW008042 (Alison K. Criss) and T32 AI007046 (M. Brittany Johnson).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 21 February 2011; paper pending published: 16 March 2011; accepted: 31 March 2011; published online: 13 April 2011.

Citation: Johnson MB and Criss AK (2011) Resistance of *Neisseria gonorrhoeae* to neutrophils. *Front. Microbio.* 2:77. doi: 10.3389/fmicb.2011.00077

This article was submitted to *Frontiers in Cellular and Infection Microbiology*, a specialty of *Frontiers in Microbiology*.

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