Resistance of *Neisseria gonorrhoeae* to non-oxidative killing by adherent human polymorphonuclear leucocytes

Alison K. Criss,† Ben Z. Katz and H. Steven Seifert

1Department of Microbiology-Immunology, Northwestern University Feinberg School of Medicine, Chicago, IL 60611, USA.
2Department of Pediatrics, Northwestern University Feinberg School of Medicine, Division of Infectious Diseases, Children’s Memorial Hospital, Chicago, IL 60614, USA.

**Summary**

Symptomatic infection with *Neisseria gonorrhoeae* (Gc) is characterized by abundant neutrophil (PMN, polymorphonuclear leucocyte) influx, but PMNs cannot clear initial infection, indicating that Gc possess defences against PMN challenge. In this study, survival of liquid-grown Gc was monitored after synchronous infection of adherent, interleukin 8-treated human PMNs. 40–70% of FA1090 Gc survived 1 h of PMN exposure, after which bacterial numbers increased. Assays with bacterial viability dyes along with soybean lectin to detect extracellular Gc revealed that a subset of both intracellular and extracellular PMN-associated Gc were viable. Gc survival was unaffected in PMNs chemically or genetically deficient for producing reactive oxygen species (ROS). This result held true even for OpaB*+* Gc, which stimulate neutrophil ROS production. Catalase- and RecA-deficient Gc, which are more sensitive to ROS in vitro, had no PMN survival defect. *recN* and *ngo1686* mutant Gc also exhibit increased sensitivity to ROS and PMNs, but survival of these mutants was not rescued in ROS-deficient cells. The *ngo1686* mutant showed increased sensitivity to extracellular but not intracellular PMN killing. We conclude that Gc are remarkably resistant to PMN killing, killing occurs independently of neutrophil ROS production and Ngo1686 and RecN defend Gc from non-oxidative PMN antimicrobial factors.

**Introduction**

*Neisseria gonorrhoeae* (the gonococcus, Gc) is an obligate human bacterial pathogen and the sole cause of the sexually transmitted infection gonorrhea. The World Health Organization estimates that 62 million cases of gonorrhea occur worldwide every year (WHO, 2001). Gc infects otherwise healthy, immunologically competent individuals, resulting in urethritis or cervicitis and potentially leading to reproductive tract scarring and sterility (Hook and Holmes, 1985). Symptomatic infection elicits a potent innate immune response at host mucosal surfaces that is characterized by the abundant influx of polymorphonuclear leucocytes (PMNs), the main component of the mucopurulent discharge of gonorrheal disease. PMN recruitment and activation is co-ordinated by chemokines such as IL-8 that are released by the infected mucosa (Ramsey et al., 1995; Christodoulides et al., 2000; Burg and Pilinger, 2001; Fichorova et al., 2001; Harvey et al., 2002). Although PMNs are normally capable of phagocytosing and killing a variety of microorganisms (Burg and Pilinger, 2001), gonorrhoeal secretions contain viable, culturable bacteria (Hook and Holmes, 1985). This observation implies that Gc have evolved strategies to evade or subvert PMN killing, contributing to the organism’s virulence potential.

Although Gc must possess some capacity for withstanding PMN challenge, studies undertaken *in vitro* to measure how well Gc survive after exposure to PMNs have not yielded consistent results (reviewed in Shafer and Rest, 1989). Some of the discrepancies may be attributed to physiological differences in PMNs in different assay systems: for instance, infection of PMNs in suspension in balanced salt solutions compared with PMNs adherent to collagen-coated surfaces in complete media (Rest and Speert, 1994, Simons et al., 2005). Other reported survival differences may relate to how effectively PMNs phagocytose Gc. PMN phagosomes fuse with granules and lysosomes, creating a phagolysosome that
is microbicidal for many organisms, including Gc (Densen and Mandell, 1978, Borregaard and Cowland, 1997, Hampton et al., 1998; Lee et al., 2003). Gc that express opacity-associated (Opa) proteins on their surface, which engage PMN carboxyembryonic antigen-related cellular adhesion molecule (CEACAM) receptors, are more readily phagocytosed and killed by PMNs than Opa-negative (Opa-) bacteria (King et al., 1978; Virji and Heckels, 1986, Fischer and Rest, 1988, Hauck et al., 1998). In contrast, intact Gc have been observed inside exudatous PMNs, and there is indirect evidence for Gc replication in PMN phagosomes (Ovcinnikov and Delekorskij, 1971, Evans, 1977; Casey et al., 1979; Apicella et al., 1996; Simons et al., 2005). Moreover, phagocytosis may not be a prerequisite for bacterial killing. PMNs can also kill extracellular microorganisms, via granule exocytosis and the release of DNA-containing neutrophil extracellular traps (Faurschou and Borregaard, 2003, Brinkmann et al., 2004), but survival of extracellular Gc during PMN infection has not been analysed.

Activated PMNs mount an oxidative burst via the NADPH oxidase enzyme, resulting in the production of bactericidal reactive oxygen species (ROS) including superoxide, hydrogen peroxide and hypochlorous acid (Hampton et al., 1998; Roos et al., 2003; Fang, 2004). PMN oxidative killing is critical for host defence, as individuals with chronic granulomatous disease (CGD), who carry mutations in NADPH oxidase, contract frequent and life-threatening infections (Johnston, 2001). Gc encode a multitude of antioxidant defences, including catalase, superoxide dismutase, peroxidases and a manganese-dependent ROS quenching system (reviewed in Seib et al., 2004), and it has been assumed that these defences have evolved in part to protect Gc from oxidative damage caused by PMNs. In support of this assumption, our laboratory recently showed that Gc upregulate the expression of 75 genes after exposure to hydrogen peroxide, and the products of two of these genes, recN and ngo1686, protect Gc from killing by both ROS and PMNs (Stohl et al., 2005). However, there is also evidence that PMNs kill Gc primarily by mechanisms independent of the oxidative burst. PMNs maintained in anoxic conditions or from CGD patients retain antigonococcal activity (Rest et al., 1982; Frangipane and Rest, 1992), and Gc carrying targeted deletions of one or more antioxidant gene products survive no differently after exposure to collagen-adherent PMNs than the wild-type parent (Seib et al., 2005). Moreover, we recently reported that liquid-grown Opa- Gc can suppress ROS production in human PMNs, suggesting that during Gc infection PMNs might only have non-oxidative antimicrobial factors at their disposal (Criss and Seifert, 2008). Well-defined non-oxidative antimicrobial factors of PMNs include degradative enzymes, such as cathepsins and lysozyme, and nonenzymatic cationic antimicrobial peptides like cathelicidins and defensins (Spitznagel, 1990; Levy, 2004). Gc are resistant to defensins at levels >0.2 mg ml⁻¹ and susceptible to killing by varying concentrations of cathepsins, the cathelicidin LL-37 and the model antimicrobial compound polymyxin B (Guymon et al., 1982; Shafer et al., 1986a; b; Qu et al., 1996). Gc encode two efflux pump systems that provide resistance to cationic antimicrobial peptides in vitro and facilitate infection of the murine genital tract, showing that some host antibacterial action is mediated through antimicrobial peptides (Shafer et al., 1998; Lee and Shafer, 1999, Jerse et al., 2003).

In this study we examined Gc survival after exposure to attached, IL-8-primed PMNs as a model for phagocytes that have migrated to the inflamed urogenital tract. We found that a sizable percentage of the bacterial inoculum survived early exposure to PMNs and both intracellular and extracellular Gc remained viable during PMN infection. PMN killing of Gc was completely independent of ROS production, revealed with bacterial mutants lacking key antioxidant genes and PMNs genetically and chemically inhibited from generating ROS. Surprisingly, the Ngo1686 and RecN gene products, which were previously shown to defend Gc from ROS in vitro, also protected Gc from non-oxidative PMN killing. Thus, PMNs primarily direct antimicrobial factors against Gc during infection that operate independently of the oxidative burst, but Gc are well equipped to handle this onslaught.

**Results**

**Adherent, IL-8-primed PMNs kill a subset of the Gc inoculum**

A previously published procedure was adapted for measuring Gc survival after exposure to primary human PMNs (Stohl et al., 2005). PMNs isolated from venous blood were allowed to adhere to tissue culture-treated plastic in the presence of IL-8. Unopsonized, piliated, predominantly Opa- Gc of strains FA1090 and MS11 were synchronously presented to PMNs at a multiplicity of infection of 1–5 bacterial colony forming units (cfu) per PMN. The bacteria were cultured in rich liquid medium using a procedure that ensures that the majority of bacteria are actively growing and at mid-logarithmic phase at the time of infection (Criss and Seifert, 2008). At various times post infection, PMNs were lysed and viable bacteria enumerated by plating and colony count.

This assay revealed that Gc infection of PMNs was biphasic. In the initial killing phase, 40–70% of the strain FA1090 Gc inoculum was recovered from PMNs after 30 min of infection (Fig. 1A, solid line), and no further reduction in bacterial survival was observed after 60 min. Similar results were obtained for Gc of strain MS11 (data not shown). PMN killing capacity for Gc varied among
donors and when PMNs were isolated from the same donor on different days (data not shown). Therefore, all the results presented are obtained with PMNs from one donor on a single day, but the survival profiles are representative of several experiments performed with PMNs from different donors. Importantly, the decline in Gc survival was due to bacterial interaction with PMNs, as Gc underwent modest growth in the infection medium without PMNs (Fig. 1A, dotted line). In the following recovery phase, the cfu associated with PMNs began to increase at 60 min post infection and continued to do so for >3 h, with an average doubling time of ~60 min (Fig. 1A, solid line), which is similar to the doubling time measured for Gc on rich medium (Tobiason and Seifert, 2006). There was no difference in Gc survival after PMN exposure when the bacteria were opsonized with autologous human serum (data not shown). These results show that Gc are partly susceptible to PMN antimicrobial activities and that this sensitivity only persists for short times following bacterial interaction with PMNs.

To examine whether PMNs were binding and internalizing the predominantly Opa- Gc in this assay system, the association of bacteria with PMNs was monitored by differential immunofluorescence, where infected cells were exposed to an anti-Gc antibody followed by different fluorescent secondary antibodies before and after PMN permeabilization. Extracellular bacteria appear red and yellow; intracellular Gc appear green. 47% of the cell-associated Gc were intracellular after 30 min.

C. Gc expressing CEACAM-binding OpaB survive less well after PMN exposure. PMNs were infected with FA1090 Gc that either lacked expression of all Opa proteins (Opa−; solid line) or expressed OpaB (OpaB+; dotted line). The average cfu ± SEM recovered at 30 and 60 min post infection from PMN lysates are expressed as a percentage of the Gc adherent at the start of the experiment (t0). Asterisk, P < 0.01 between Opa− and OpaB+ Gc at matched time points (Student’s two-tailed t-test).
Thus, Opa expression was not required for unopsonized Gc to attach to and be internalized by PMNs in this assay system, in contrast to previous reports (Rest et al., 1982). However, FA1090 expression of the CEACAM-binding OpaB enhanced bacterial association with PMNs (Fig. S1), and OpaB+ Gc survived less well than Opa− bacteria after exposure to PMNs (Fig. 1C). We conclude that even though Opa+ bacteria are more efficiently phagocytosed by PMNs, PMNs internalize and kill a fraction of Opa− Gc, enabling us to use Opa− Gc to study bacterial survival during PMN infection.

**Differential survival of Gc adherent to and internalized by PMNs**

Having found that adherent, IL-8-treated human PMNs kill a subset of Opa− Gc, we sought to define the survival of attached and intracellular bacterial populations during PMN infection. Transmission electron micrographs of adherent PMNs infected with unopsonized, Opa− Gc for 30 min verified that bacteria were closely apposed to the PMN surface and phagocytosed into vacuoles (Fig. 2A). The majority of the extracellular bacteria appeared electron-dense, intact and potentially viable, but the occasional electron-lucent, non-viable bacterium was observed (Fig. 2A, asterisk). Phagosomes containing Gc were often in close proximity to cytoplasmic granules, with evidence of phagosome–granule fusion (Fig. 2A, inset), and often contained electron-lucent bacteria (Fig. 2A, white arrows). These images suggested that PMNs can kill intracellular Gc and imply that killing is attributable to granule components, in agreement with previous reports (Ovcinnikov and Delektorskij, 1971; Densen and Mandell, 1978). However, other internalized bacteria appeared electron-dense (Fig. 2A, black arrows), suggesting that they might be viable. To test whether phagocytosis was required for PMNs to kill Gc, bacterial survival was measured in PMNs treated with the actin-depolymerizing compound cytochalasin D (CD). Although CD treatment significantly increased the number of viable Gc recovered from infected PMNs, bacterial viability was still reduced relative to Gc cultured in the absence of PMNs (Fig. 2B). These results suggest that not all PMN killing of Gc occurs intracellularly and, conversely, some Gc may survive within PMNs.

To directly measure the viability of both extracellular and internalized Gc during PMN infection, we developed a fluorescence microscopy assay utilizing a commercially available bacterial viability kit (BacLight LIVE/DEAD Viability Kit, Invitrogen). Viable, intact bacteria incorporate the green fluorescent SYTO9 dye, while bacteria with compromised membranes are permeable to propidium iodide (PI) and fluoresce red. In preliminary experiments, addition of 0.1% saponin to the dye-containing medium was necessary for the fluorescent dyes to equally access the intracellular bacteria but had no effect on bacterial viability itself (data not shown). In order to discriminate intracellular from extracellular bacteria, Gc-infected PMNs were incubated with Alexa Fluor 647-coupled soybean

Fig. 2. PMNs have antignonococcal activity against intracellular and extracellular bacteria.

A. Electron microscopy reveals that some Gc inside PMNs are non-viable. Adherent, IL-8-treated PMNs were infected with Gc for 30 min, then fixed and processed for thin-section transmission electron microscopy. Asterisk indicates a non-viable extracellular bacterium. White arrows indicate non-viable intracellular bacteria, while black arrows denote intracellular bacteria that retain electron density, suggesting that they have retained viability. Inset is a magnified view of the boxed region of the micrograph, depicting a Gc-containing phagosome in proximity to numerous cytoplasmic granules.

B. CD-treated PMNs are reduced but not inhibited in their ability to kill Gc. PMNs were treated with CD (+CD, dashed line) or DMSO carrier (−CD, solid line), then infected with FA1090 Gc. Survival was calculated as in Fig. 1A and is compared with Gc maintained in infection medium without PMNs and in the presence of an equal volume of DMSO (no PMN, dotted line). Asterisk, P < 0.05 between CD- and DMSO-treated PMNs at matched time points (Student’s two-tailed t-test). Gc maintained in the absence of PMNs survived significantly better than Gc exposed to either CD- or DMSO-treated PMNs (P < 0.025 for matched time points; Student’s two-tailed t-test).
agglutinin (SBA) before treatment with saponin. This lectin was reported to bind certain isolates of Gc (Allen et al., 1980; Schalla et al., 1985), and we determined that it decorated the surface of strain FA1090 Gc but weakly interacted with PMNs (Fig. 3A). Cells were not fixed with primary aldehydes or alcohols, which we found permeabilized the bacteria to PI. Single ~130-nm-thick optical slices were taken through each imaging field by confocal laser scanning microscopy. From the collected images, viability was scored by counting the intracellular (SBA−) and extracellular (SBA+) bacterial particles associated with PMNs and calculating the percentage of viable (PI−) bacteria in each of these populations.

As expected from growth curves with FA1090 Gc, >95% of bacteria grown in rich liquid medium were PI-negative, and this percentage did not change when Gc were cultured in infection medium without PMNs (Fig. 3A). In contrast, overall bacterial viability declined after 30 min of infection to levels qualitatively similar to those measured in Fig. 1A. In three independent experiments, the percentage of total PI+/SBA−, PMN-associated bacteria averaged 23% (Fig. 3B). If PMNs were exclusively killing internalized Gc, only two populations of bacteria should be detected: PI+/SBA− (intracellular non-viable) and PI−/SBA+ (extracellular viable). Instead, all four possible combinations of lectin and viability stains were detected in Gc associated with PMNs after 30 min (Fig. 3B), yielding two surprising observations. First, greater than half (58%) of the intracellular Gc were viable (PI−/SBA−). Second, the viability of extracellular Gc was reduced in comparison...
with bacteria kept in infection medium without PMNs (23% versus <5% respectively). These results show that PMNs kill both intracellular and extracellular Gc, but a subset of bacteria survive in both locations.

After 120 min of infection, the percentage of viable Gc associated with PMNs did not change significantly, and we calculated that approximately 81% of the extracellular bacteria and 41% of the intracellular bacteria were viable (Fig. 3C). However, the total number of Gc associated with PMNs increased more than twofold over what was measured at 30 min, from an average of 3.8 cfu per PMN to 9.0 cfu per PMN, indicating that bacterial recovery was occurring (Fig. 3E). Moreover, >60% of the bacteria associated with PMNs during the recovery phase were intracellular, leading to a sizable increase in the number of viable intracellular Gc at this time (Fig. 3E). Some PMNs contained large numbers of viable intracellular bacteria that clustered in the same region of the cell (arrow, Fig. 3C), suggestive of intracellular bacterial replication in PMNs. These observations indicate that Gc are replicating in association with PMNs, with many of the bacteria remaining viable, as suggested from studies of PMNs from gonorrheal exudates (Ovcinnikov and Delektorskij, 1971; Apicella et al., 1996).

Our laboratory previously constructed a loss-of-function mutation in ngo1686, and this mutant’s survival was significantly reduced after PMN infection (Stohl et al., 2005). We did not measure any difference in the ability of ngo1686 to associate with or be internalized by PMNs compared with the FA1090 parent strain (data not shown). When the direct viability fluorescence assay was applied to ngo1686-infected PMNs, we observed that approximately 55% of all ngo1686 Gc were PI+ after 30 min exposure to PMNs, a substantial increase over the FA1090 parent. This was specifically due to a decrease in viability of the extracellular ngo1686 bacteria (Fig. 3D and F). These results validate the use of the direct viability fluorescence assay to detect modest changes (twofold to threefold) in bacterial viability during PMN infection and demonstrate that ngo1686 mutant bacteria are more sensitive to PMN killing mechanisms directed extracellularly.

Taken together, these results show that while many Gc internalized by PMNs are killed within phagosomes, others survive and may be able to proliferate therein. PMNs also kill a fraction of the bacteria adherent to the PMN surface, but the majority of extracellular bacteria survive. We conclude that Gc–PMN interactions are complex, with non-viable and viable populations of bacteria arising both intracellularly and extracellularly.

**PMNs kill Gc exclusively by non-oxidative means**

Given the ability of a subset of Gc to survive PMN infection, we sought to define which antimicrobial activities of PMNs were being directed against Gc during infection. Our laboratory previously defined the Gc transcriptional response to H2O2 and hypothesized that these changes would help defend the bacteria from PMN attack, assuming that PMNs generate ROS like H2O2 in their antigonococcal arsenal (Stohl et al., 2005). As evidence for this hypothesis, products of two of the genes upregulated in response to H2O2, ngo1686 and recN, protect Gc from **in vitro** PMN killing (Stohl et al., 2005). Additionally, Gc treated with sublethal concentrations of H2O2 survived better during prolonged incubation with PMNs than untreated bacteria (Fig. 4A). Accordingly, we expected that other mutants that are more sensitive to ROS should also be more sensitive to PMN challenge. We therefore examined the PMN survival profile of two mutant bacterial strains, one devoid of catalase (encoded by the kat gene) and the other deficient for the recombinase recA. Both mutants are killed more readily by ROS (Johnson et al., 1993; Soler-Garcia and Jerse, 2004; Stohl et al., 2005; Stohl and Seifert, 2006). However, each mutant survived as well as its isogenic parent after incubation with PMNs (Fig. 4B and C). Results with catalase-deficient Gc were validated using two independently derived mutants (Fig. 4B), and ngo1686 served as a control for the ability of these PMNs to kill another Gc mutant (Fig. 4C). These results show a lack of correlation between Gc mutants’ sensitivity to ROS and to PMN killing, in concordance with findings from other groups (Seib et al., 2005; Soler-Garcia and Jerse, 2007; Wu et al., 2009) and our recent report that liquid-grown Gc suppress the PMN oxidative burst (Cris and Seifert, 2008).

To directly examine whether ROS production played any role in the antigonococcal activity of PMNs, phagocytes were treated with the NADPH oxidase inhibitor diphenylidylenediiodonium hydrochloride (DPI). Pretreatment with 10 μM DPI completely blocked the ability of PMNs to generate an oxidative burst in response to phorbol ester stimulation (Fig. 5A) and rescued the PMN survival defect of a non-pigmented *Staphylococcus aureus* mutant that is extremely sensitive to oxidative PMN killing (Fig. 5B) (Liu et al., 2005). In contrast, Gc survival was unaffected in DPI-treated PMNs (Fig. 5C, ‘FA1090’ bracketed lines). Moreover, the DPI-treated PMNs were still able to kill ngo1686 (Fig. 5C, ‘ngo1686’ bracketed lines) and recN (Fig. 5D) mutants significantly better than the isogenic parent strain. Survival of parental or ngo1686 Gc was not altered in PMNs treated with ABAH, which inhibits the myeloperoxidase enzyme that produces hypochlorous acid, or L-NMMA, which inhibits nitric oxide synthase-mediated production of reactive nitrogen species (Fig. S1). Therefore, PMNs chemically inhibited from producing reactive oxygen or nitrogen species retain the ability to kill Gc. Even though the ngo1686 and recN mutants are more susceptible to ROS
in vitro, this does not account for their increased sensitivity to PMN killing.

Although we previously found that liquid-grown Opa–Gc fail to induce the PMN oxidative burst, OpaB+ FA1090 Gc promoted a minor burst in these cells (Criss and Seifert, 2008). This raised the possibility that the decrease in OpaB+ Gc survival after PMN infection, as compared with isogenic Opa– bacteria, was attributable to PMN-derived ROS. However, no difference in OpaB+ Gc survival was observed in PMNs pretreated with DPI compared with untreated controls (Fig. 6). This result indicates that even in conditions where PMNs mount an oxidative burst (i.e. OpaB+ infection), ROS generation is dispensable for PMN antigonococcal activity.

To independently confirm these results without using pharmacological inhibitors, Gc survival was measured in adherent, IL-8-treated PMNs isolated from two unrelated individuals with CGD (Johnston, 2001). These PMNs were completely incapable of mounting the oxidative burst (see Experimental procedures). CGD PMNs retained the ability to kill FA1090 Gc, as 30–50% of the cfu present at the start of the experiment were recovered after infection (Fig. 7). This observation is in agreement with a previous report with human CGD PMNs and a different Gc strain (Rest et al., 1982). However, given the variation in the ability of wild-type PMNs to kill Gc, this result was not sufficient to convince us that CGD PMNs were as effective as their wild-type counterparts in combating Gc infection. Therefore, we used the ngo1686 mutant as readout for CGD PMN antigonococcal activity, reasoning that if survival of the mutant was restored to wild-type levels, it would indicate that PMN-derived ROS were necessary to combat Gc infection. Instead, we found that the mutant remained significantly more sensitive than the parental strain to CGD PMNs (Fig. 7), demonstrating that Ngo1686 protects Gc from non-oxidative PMN antimicrobial mechanisms. Taking the results with CGD PMNs in combination with the DPI studies and with survival profiles of Gc antioxidant mutants, we conclude that adherent, chemokine-treated human PMNs kill Gc by mechanisms independent of the oxidative burst.

**Discussion**

The long-term association of Gc within the human population reflects its successful adaptation to life exclusively in the human urogenital tract, including withstanding the potent PMN-driven immune response to infection. In this work, we provide direct evidence for survival of a high percentage of Gc after exposure to primary human PMNs in vitro and demonstrate that the bacteria can remain intact within PMNs for hours following infection. We show that the minor amount of Gc clearance by PMNs is completely independent of phagocyte NADPH oxidase activity, even

---

**Fig. 4.** Gc with diminished antioxidant defences vary in their survival to PMNs.
A. Pre-exposure of Gc to ROS enhances their survival to PMNs. FA1090 Gc were left untreated (solid line) or treated with 1 mM H2O2 prior to infection of adherent PMNs. H2O2-treated FA1090 Gc survived better after PMN infection than untreated bacteria (asterisks, $P < 0.05$, Student’s two-tailed t-test). B. Gc catalase mutants have no survival defect during PMN infection. PMNs were infected with FA1090 Gc (solid line) or one of two catalase-deficient mutants (kat::aph, dashed line; or kat::Ω [Spc]), dotted line). No significant difference in survival was observed between FA1090 Gc and either of the mutants. C. A recA mutant has no survival defect during PMN infection. PMNs were infected with FA1090 Gc (solid line) or isogenic mutants in recA (dashed line) or ngo1686 mutant (dotted line). No significant difference in survival was observed between FA1090 Gc and the recA mutant, while the ngo1686 mutant survived significantly less well than the FA1090 parent (asterisks, $P < 0.05$, Student’s two-tailed t-test).
under conditions that stimulate the oxidative burst (infection with OpaB+ Gc), and we define that the Ngo1686 and RecN gene products protect Gc from non-oxidative killing by PMNs. Coupled with our recent report that liquid-grown Gc can suppress the oxidative burst of PMNs (Criss and Seifert, 2008), we conclude that Gc have evolved sophisticated ways to survive and proliferate in the presence of PMNs and antimicrobial factors produced by PMNs and other cells in the urogenital tract.

PMNs found in gonorrheal exudates are chemokine-primed and adherence-competent, a consequence of their migration from the bloodstream to the mucosal epithelium. Our goal was to establish an assay that recapitulated this environment in vitro using attached, IL-8-treated PMNs. PMNs cultured in these conditions were fully functional, defined by their ability to generate ROS in response to phorbol ester stimulation and to kill the pathogen S. aureus in a NADPH oxidase-sensitive manner (see Fig. 5). When PMNs were presented with

Fig. 5. Treatment of PMNs with the NADPH oxidase inhibitor DPI does not affect Gc survival.

A. DPI abrogates the ability of PMNs to generate ROS after phorbol ester stimulation. PMNs were pretreated with DPI (dashed line) or an equal volume of DMSO carrier (solid lines). PMNs were left unstimulated (solid grey line) or stimulated with the phorbol ester PMA, and luminol-dependent chemiluminescence was recorded over 1 h. DPI-treated PMNs generated no more ROS than unstimulated PMNs.

B. DPI treatment rescues the survival defect of a S. aureus mutant that is extremely sensitive to ROS. After pretreatment with DPI (dashed line) or an equal volume of carrier (solid line), PMNs were infected with S. aureus lacking carotenoid antioxidant pigment due to insertional inactivation of crtM. crtM S. aureus survived significantly better in DPI-treated PMNs than control PMNs (asterisks, P < 0.005, Student's two-tailed t-test).

C. DPI treatment does not rescue the survival defect of ngo1686 Gc in PMNs. PMNs were pretreated with DPI (dashed lines) or DMSO (solid lines), then infected with FA1090 Gc (diamonds) or the ngo1686 mutant (squares). FA1090 Gc survived better in DPI-treated PMNs than control PMNs (asterisk, P < 0.05, Student's two-tailed t-test) regardless of whether the PMNs were treated with DPI or not.

D. DPI treatment does not rescue the survival defect of recN Gc in PMNs. PMNs from a different donor on a different day were pretreated with DPI (dashed lines) or DMSO (solid lines), then infected with FA1090 Gc (diamonds) or the recN mutant (triangles). FA1090 Gc survived significantly better in PMNs than the recN mutant (asterisks, P < 0.025, Student's two-tailed t-test); DPI treatment had no significant effect on the survival of recN Gc.

Fig. 6. DPI treatment does not enhance the survival of OpaB+ Gc in PMNs. PMNs were pretreated with DPI (dashed line) or DMSO (solid line), then infected with FA1090 Gc expressing the CEACAM-binding OpaB. DPI treatment had no significant effect on the survival of OpaB+ Gc.
Gc, large numbers of bacteria were engulfed, as has been observed in PMNs collected from infected individuals. Yet in contrast to previous reports, phagocytosis of predominantly Opa− bacteria occurred in the absence of serum opsonization. Explanations for this discrepancy include that adherent PMNs may upregulate the cell surface presentation of additional receptors that recognize Gc or that these cells are inherently more phagocytic for small 0.5–1 μm particles like Gc. Notably, under these assay conditions, PMN killing mechanisms were only effective against Gc for the first hour of coinoculation, and Gc were relatively resistant to these mechanisms. This result may indicate that PMNs are more adept at killing a subpopulation of Gc that somehow differ from the majority of bacteria, or alternatively that PMN killing of Gc is stochastic. It is important to note that this assay reports on the viability of both intracellular and extracellular bacteria, a necessity of the system because treatment of PMNs with gentamicin yielded no recoverable cfu, as was found by Simons et al. (2005). Instead of being a limitation of the system, the absence of gentamicin afforded us the opportunity to monitor the viability of both attached and internalized bacteria, as will be discussed below. After the first hour of infection, we found that the number of Gc associated with PMNs increased, without any observable effect on PMN integrity. We are currently assessing if the survivors have undergone pheno- typic changes that render them more resistant to PMNs or if PMNs lose antimicrobial activities after prolonged infection with Gc.

PMNs are generally considered to kill bacteria and fungi by internalizing them into phagosomes that mature into degradative phagolyosomes, and Densen and Mandell demonstrated that the PMN phagosome is bactericidal for Gc (Densen and Mandell, 1978; Lee et al., 2003). However, this does not agree with the observation that phagocytes in gonorrheal exudates contain intact bacteria or with a recent report by Simons et al. that adherent PMNs infected in vitro with Gc contain intact (electron-dense) bacteria several hours post infection (Ovcinnikov and Delektorskij, 1971; Evans, 1977; Apicella et al., 1996; Simons et al., 2005). These divergent results suggest that there are locations or compartments where the antgonococcal activity of PMNs is not effective. Prior to this work, there were no reports on the viability of extracellular Gc in close proximity to PMNs. The fluorescence-based assay we developed for measuring the viability of all Gc associated with PMNs is more direct and more sensitive than observing electron-dense bacteria by thin-section electron microscopy, and should be applicable not only to Gc mutants of interest but also other bacterial species. Results from this assay demonstrated that many Gc internalized by PMNs are killed, as expected, but also showed that a sizable population of bacteria reside inside PMNs and increase in number over time. Also as expected, the majority of PMN-associated Gc were viable, but the percent of extracellular bacteria that were no longer viable was significantly greater than found in the initial Gc inoculum. These dead extracellular bacteria were typically in close apposition to the PMN membrane, suggesting that killing occurs by antimicrobial factors released either by granule exocytosis or neutrophil extracellular traps. Notably, the ngo1686 mutant was specifically more sensitive to extracellular killing by PMNs, suggesting that the mutant either triggers greater extracellular release of antimicrobial factors from PMNs or is inherently more sensitive to these factors.

Our observations show conclusively that the phagocyte oxidative burst is dispensable for PMN killing of Gc. From the host side, DPI-treated PMNs and PMNs from individuals with CGD retained the ability to kill the bacteria, as was reported for suspension, unprimed PMNs maintained under anoxic conditions and from CGD patients (Rest et al., 1982; Frangipane and Rest, 1992). These results are consistent with the fact that individuals with CGD are not disproportionately infected with Neisseria species, specifically N. meningitidis, which colonizes the nasopharynx and occasionally causes invasive disease (Johnston, 2001). Additionally, Gc colonize the genital tract of CGD-like mice lacking NADPH oxidase activity.

© 2009 Blackwell Publishing Ltd, Cellular Microbiology, 11, 1074–1087
(deletion of gp91phox) no differently from wild-type C57/BL6 mice (Wu et al., 2009). From the bacterial side, kat-and recA-deficient bacteria were no more sensitive to PMN killing than their wild-type counterparts, corroborating recent results with a variety of Gc antioxidant mutants in different assay settings (Seib et al., 2005; Soler-Garcia and Jerse, 2007; Wu et al., 2009). Intriguingly, mutants in the ngo1686 and recN genes, which show increased sensitivity to ROS in vitro, were more susceptible to PMN killing in an NADPH oxidase-independent manner. The mutants attached to and were internalized by PMNs as readily as the wild-type parent strain, and like the Opa–FA1090 parent, the mutants did not elicit any detectable oxidative burst in PMNs (data not shown). Therefore, we conclude that the Ngo1686 and RecN gene products enhance bacterial resistance not only to ROS but also to non-oxidative PMN antimicrobial factors. Ngo1686 shares sequence similarity with zinc-dependent proteases and may have the ability to degrade antimicrobial peptides or proteins, analogously to a surface metalloprotease of L. donovani (Kulkarni et al., 2006). As the ngo1686 mutant was significantly more susceptible to extracellular killing by PMNs, the Ngo1686 protein may specifically proteolyse antimicrobial factors released by PMN exocytosis. The mechanism by which RecN, which is important for recombinational DNA repair in Gc (Skaar et al., 2002), protects Gc from non-oxidative PMN killing, is less clear. However, some cationic antimicrobial peptides can inhibit bacterial nucleic acid synthesis (reviewed in Brogden, 2005), raising the possibility that a recN mutant, which is already more sensitive to DNA damage than its wild-type counterpart, may be less able to withstand additional damage (to DNA or to other targets) inflicted by non-oxidative antimicrobial agents. Future studies will continue to explore the non-oxidative mechanisms directed by PMNs against Gc and the Gc gene products, including Ngo1686 and RecN, which defend against them.

Our studies have shown that PMNs kill Gc independently of ROS production and live Gc suppress the PMN oxidative burst, yet Gc retain a large number of gene products in their genome that defend against ROS and upregulate expression of many of these products upon exposure to ROS. We propose that exposure of Gc to ROS vacillates with the course of infection, and Gc responds to these changes with a broad assortment of oxidative and non-oxidative defence mechanisms. During initial colonization of the urogenital tract, Gc encounter ROS produced from commensal lactobacilli and potentially epithelial cells (St Amant et al., 2002). The ROS from these sources can impede bacterial growth, but Gc, being relatively resistant to ROS, survive and multiply at the mucosal surface. Bacterial colonization is also aided by the 1–5 day delay in appearance of PMNs in the urogenital tract following infection (Cohen and Cannon, 1999). The majority of viable Gc that encounter the first wave of PMNs are actively growing and therefore capable of suppressing the phagocyte oxidative burst. However, if Gc are killed by cationic antimicrobial peptides and other factors released in the urogenital tract, or if live Gc release substantial amounts of outer membrane-derived material that is immunostimulatory, the dead bacteria and bacterial products would overcome the suppressive effect of live Gc on PMN oxidative metabolism, allowing PMNs to mount a respiratory burst (Criss and Seifert, 2008). Importantly, ROS, whether from PMNs or other sources, would signal Gc to upregulate expression of proteins such as Ngo1686 and RecN that increase bacterial resistance not only to ROS but also to non-oxidative PMN killing. By providing new insights into how Gc resist PMN clearance, these results have defined unique characteristics of the complex relationship between this obligate human pathogen and the host innate immune system, and we anticipate identifying additional pathogenic mechanisms that facilitate Gc survival, replication and spread within the human population.

## Experimental procedures

### Bacterial strains and growth conditions

The Gc used in this study were pilated, Opa–derivatives of strain FA1090 encoding pilin variant 1-81-S2 (Seifert et al., 1994) and strain MS11 encoding pilin variant VD300 (Koomey et al., 1987). Bacteria were maintained on Gonococcal Medium Base (Difco) plus Kellogg’s supplements (Kellogg et al., 1963) and routinely grown for 20 h at 37°C in 5% CO2. Viable, exponentially growing Gc were obtained from successive rounds of bacterial growth in rich liquid medium as described (Criss and Seifert, 2008). The FA1090 1-81-S2 recA4::telM, ngo1686::ermC, recN::ermC and kat::aph mutants and the Opa+ derivative of FA1090 1-81-S2 have been described (Seifert, 1997; Skaar et al., 2002; Soler-Garcia and Jerse, 2004; Stohl et al., 2005; Criss and Seifert, 2008). The Gc strain 2374 kat::\(\Omega_{W}^{spc}^{+}\) mutation was obtained from S. Johnson (United States Centers for Disease Control) (Johnson et al., 1993). The \(\Omega_{W}^{spc}^{+}\) mutation was introduced into FA1090 1-81-S2 Gc by natural transformation, colonies resistant to 40 \(\mu\)g ml\(^{-1}\) spectinomycin were isolated, and replacement of the parental kat allele with \(\Omega_{W}^{spc}^{+}\) was confirmed by PCR and Southern blotting with a kat-specific gene probe. Opa protein expression profiles were determined by immunoblotting bacterial lysates with a panel of monoclonal and polyclonal antibodies (obtained from J. Cannon, University of North Carolina and A. Jerse, USUHS) according to published methods (Black et al., 1984). The pilE genes of the Gc strains were sequenced as described (Seifert et al., 1994) to confirm retention of the parental pilin variant.

The S. aureus crtM carotenoid-deficient mutant was obtained from V. Nizet (UCSD). S. aureus were grown on Todd-Hewitt agar (Difco) for 16 h. For each experiment, single colonies were inoculated into Todd-Hewitt broth (Difco) and grown with rotation at 37°C for 48 h, as previously described (Liu et al., 2005). Prior to infection, S. aureus were opsonized in 10% autologous human serum for 20 min at 37°C.
PMN donors

Heparinized venous blood was obtained from consented healthy volunteers and consented individuals with CGD undergoing routine examination at the outpatient clinic of the Division of Infectious Diseases, Children’s Memorial Hospital, Chicago, following a protocol approved by the Children’s Memorial Research Center and Northwestern University Institutional Review Board. Individuals with CGD did not present with any infections at the time of venipuncture.

CGD donor #1 is a gp91phox-deficient male. The affected CYBB gene has a frameshift mutation of exon 3 (a C inserted after G169) resulting in a premature stop codon in exon 5. PMN extracts showed no cytchrome b558 present and no superoxide produced after PMA stimulation. A slide nitroblue tetrazolium test showed that 0% of the PMNs underwent a respiratory burst.

The genetic deficiency in CGD donor #2, also male, has not been defined. A whole-blood flow cytometry assay was used to demonstrate that PMNs from donor #2 produced no respiratory burst (O'Gorman and Corrochano, 1995).

PMN isolation

Dextran-sedimented PMNs were purified on a Ficoll-Hypaque gradient as previously described (Stohl et al., 2005). PMNs were resuspended at 1 × 10⁷ cells ml⁻¹ in Dulbecco’s PBS (without calcium and magnesium; Mediatech) containing 0.1% dextrose and kept on ice until use. PMN preparations routinely contained > 95% PMNs, assessed morphologically by phase-contrast microscopy, and were > 99% viable, monitored by trypan blue exclusion.

Adherent PMN assay

The adherent, IL-8 treated PMN assay with Gc was performed as previously described (Stohl et al., 2005). All Gc retained the correct Opa phenotype throughout the course of infection.

Experiments with non-CGD PMNs were performed with five replicate PMN monolayers per time point. Where indicated, PMNs were treated with 0.1 mM 4-aminobenzoic acid hydrazide (ABAH; EMD Biosciences) to inhibit myeloperoxidase, 0.2 mM N²-methyl-L-arginine acetate salt (L-NMMA; Sigma) to inhibit nitric oxide synthase or 10 μM DPI (Sigma) to inhibit NADPH oxidase, then washed from the cells prior to infection. To block phagocytosis, 10 μg ml⁻¹ CD in DMSO (Sigma) was added to PMNs 10 min prior to infection and was kept in the medium for the duration of the experiment. DMSO treatment alone did not affect Gc interaction with PMNs (data not shown). In H₂O₂ presaturation experiments, exponential-phase liquid Gc cultures were diluted 1:10 in GCBL containing H₂O₂ (Sigma) at a final concentration of 1 mM and incubated at 37°C with rotation for 30 min. This concentration of H₂O₂ did not significantly reduce Gc survival (data not shown). Cultures were then treated with bovine catalase (final concentration of 10 μg ml⁻¹; Sigma) to degrade residual H₂O₂, and bacteria were washed into infection medium and added to PMNs.

Results shown for non-CGD PMNs are representative of at least three experiments, with each experiment using PMNs from a different donor. Experiments with CGD PMNs were performed once per donor, with each time point measured in triplicate, due to the limited frequency with which the individuals with CGD attended the outpatient clinic and restrictions on the amount of blood collected per individual at each visit, in accordance with Institutional Review Board guidelines.

Microscopic examination of Gc internalization by PMNs

Adherent PMNs were infected with Gc as described above. 30 min post infection, PMNs were fixed in 4% paraformaldehyde (Electron Microscopy Sciences) in PBS. Internal and external bacteria were discriminated from one another using a polyclonal anti-Gc antibody (Biosource) and a differential immunofluorescence procedure as previously described (Criss and Seifert, 2006). Cells were examined using a Zeiss LSM510 confocal laser scanning microscope with a 100×, 1.4 numerical aperture objective. Each image was a single optical slice of ~130 nm through each field. Images were acquired with LSM510 operating software and processed with LSM Image Browser (Zeiss) and/or Adobe Photoshop CS3 (Adobe). In order to acquire images in an unbiased manner as possible, image fields were randomly chosen that contained Gc (e.g. had green fluorescent particles). All quantification occurred after images were acquired and processed. The number of fluorescent Gc particles associated with and internalized by each PMN were counted to arrive at an average number of Gc per PMN and the per cent internalization of Gc by PMNs.

Viability of intracellular and extracellular Gc associated with PMNs

PMNs attached to glass coverslips (Fisher) coated with 0.1% poly L-lysine (Sigma) were infected with FA1090 Gc for 30 or 120 min, or ngo1686 Gc for 30 min. PMNs were washed and incubated in 0.1 MOPS pH 7.2, 1 mM MgCl₂ (MOPS/MgCl₂) containing 5 μg ml⁻¹ Alexa Fluor 633-SBA (Invitrogen) for 10 min at room temperature for detection of extracellular bacteria (Allen et al., 1980). PMNs were then washed into MOPS/MgCl₂ containing 0.1% saponin, 60 μM of PI and 10 μM of SYTO9 and incubated at room temperature for 15 min. PI and SYTO9 are components of the BacLight Viability Kit (Invitrogen). PMNs were examined within 30 min of mounting, as recommended by the kit manufacturer, using a Zeiss LSM510 laser scanning confocal microscope. Images were acquired and processed as described above. In each experiment, 50–100 individual infected PMNs were examined per bacterial strain and time point. In order to acquire images in an unbiased manner as possible, imaging fields were randomly chosen for the presence of PMN nuclei, which appeared as PI-positive objects significantly larger than Gc (see Fig. 3B–D for examples). All quantification of bacterial internalization and viability was performed after image acquisition and processing. The per cent of PI⁺ Gc was calculated for both SBA-positive and -negative bacteria. In control experiments, heat-killed Gc inside saponin-permeabilized PMNs were accessible to both SYTO9 and PI, but saponin did not permeabilize live bacteria to PI (data not shown).

Thin-section transmission electron microscopy

PMNs attached to Thermax coverslips (Nunc) were infected with FA1090 Gc for 30 min. PMNs were washed in ice-cold 0.1 M
sodium cacodylate-HCl, pH 7.3 and fixed at 4°C in cacodylate buffer containing 2.5% glutaraldehyde. Samples were treated with 2% osmium tetroxide, dehydrated in increasing grades of ethanol and embedded in Epon resin (Electron Microscopy Sciences). 100 nm thin sections were mounted on Formvar carbon-coated grids (Electron Microscopy Sciences), negatively stained with uranyl acetate and lead citrate, and examined with a JEOL 1220 transmission electron microscope at an accelerating voltage of 60 kV. Images were acquired with a Gatan digital camera and associated software and processed with Adobe Photoshop CS3.

Luminol-dependent chemiluminescence

The ability of PMNs to generate ROS after stimulation with 1 ng ml⁻¹ PMA (Sigma) with and without DPI pretreatment was measured by luminol-dependent chemiluminescence as previously described (Criss and Seifert, 2008).

Acknowledgements

We thank Ann Jerse, Steven Johnson, Victor Nizet and George Liu for strains and reagents, and Alan Hauser, Ciara Shaver, Laurie Tudor and John Carter for assistance with venipuncture. We especially thank Stanford Shulman for facilitating access to CGD patients as well as the CGD patients for participating in this study. This work was supported by R01 AI044239, R01 055977 and R37 AI035493 to H.S.S. and F32 AI056681 and K99 TW008042 to A.K.C.

References

Allen, P.Z., Connelly, M.C., and Apicella, M.A. (1980) Interaction of lectins with Neisseria gonorrhoeae. Can J Microbiol 26: 468–474.

Apicella, M.A., Ketterer, M., Lee, F.K., Zhou, D., Rice, P.A., and Blake, M.S. (1996) The pathogenesis of gonococcal urethritis in men: confocal and immunoelectron microscopic analysis of urethral exudates from men infected with Neisseria gonorrhoeae. J Infect Dis 173: 636–646.

Black, W.J., Schwalbe, R.S., Nachamkin, I., and Cannon, J.G. (1984) Characterization of Neisseria gonorrhoeae protein II phase variation by use of monoclonal antibodies. Infect Immun 45: 453–457.

Borregaard, N., and Cowland, J.B. (1997) Granules of the human neutrophil polymorphonuclear leucocyte. Blood 89: 3503–3521.

Brinkmann, V., Reichard, U., Goosmann, C., Fauler, B., Uhlemann, Y., Weiss, D.S., et al. (2004) Neutrophil extracellular traps kill bacteria. Science 303: 1532–1535.

Brogden, K.A. (2005) Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? Nat Rev Microbiol 3: 238–250.

Burg, N.D., and Pillinger, M.H. (2001) The neutrophil: function and regulation in innate and humoral immunity. Clin Immunol 99: 7–17.

Casey, S.G., Veale, D.R., and Smith, H. (1979) Demonstration of intracellular growth of gonococci in human phagocytes using spectinomycin to kill extracellular organisms. J Gen Microbiol 113: 395–398.

Christodoulides, M., Everson, J.S., Liu, B.L., Lambden, P.R., Watt, P.J., Thomas, E.J., and Heckels, J.E. (2000) Interaction of primary human endometrial cells with Neisseria gonorrhoeae expressing green fluorescent protein. Mol Microbiol 35: 32–43.

Cohen, M.S., and Cannon, J.G. (1999) Human experimentation with Neisseria gonorrhoeae: progress and goals. J Infect Dis 179: S375–S379.

Criss, A.K., and Seifert, H.S. (2006) Gonococci exit apically and basally from polarized epithelial cells and exhibit dynamic changes in type IV pili. Cell Microbiol 8: 1430–1443.

Criss, A.K., and Seifert, H.S. (2008) Neisseria gonorrhoeae suppresses the oxidative burst of human polymorphonuclear leucocytes. Cell Microbiol 10: 2257–2270.

Densen, P., and Mandell, G.L. (1978) Gonococcal interactions with polymorphonuclear neutrophils: importance of the phagosome for bacterial activity. J Clin Invest 62: 1161–1171.

Evans, B.A. (1977) Ultrastructural study of cervical gonorrhoea. J Infect Dis 136: 248–255.

Fang, F.C. (2004) Antimicrobial reactive oxygen and nitrogen species: concepts and controversies. Nat Rev Microbiol 2: 820–832.

Fauschou, M., and Borregaard, N. (2003) Neutrophil granules and secretory vesicles in inflammation. Microbes Infect 5: 1317–1327.

Fichorova, R.N., Desai, P.J., Gibson, F.C., 3rd, and Genco, C.A. (2001) Distinct proinflammatory host responses to Neisseria gonorrhoeae infection in immortalized human cervical and vaginal epithelial cells. Infect Immun 69: 5840–5848.

Fischer, S.H., and Rest, R.F. (1988) Gonococci possessing only certain PII outer membrane proteins interact with human neutrophils. Infect Immun 56: 1574–1579.

Frangipane, J.V., and Rest, R.F. (1992) Anaerobic growth of gonococci does not alter their Opa–mediated interactions with human neutrophils. Infect Immun 60: 1793–1799.

Guymon, L.F., Esser, M., and Shafer, W.M. (1982) Pyocin-resistant lipopolysaccharide mutants of Neisseria gonorrhoeae: alterations in sensitivity to normal human serum and polymyxin B. Infect Immun 36: 541–547.

Hampton, M.B., Kettle, A.J., and Winterbourn, C.C. (1998) Inside the neutrophil phagosome: oxidants, myeloperoxidase, and bacterial killing. Blood 92: 3007–3017.

Harvey, H.A., Post, D.M., and Apicella, M.A. (2002) Immortalization of human urethral epithelial cells: a model for the study of the pathogenesis of and the inflammatory cytokine response to Neisseria gonorrhoeae infection. Infect Immun 70: 5808–5815.

Hauck, C.R., Meyer, T.F., Lang, F., and Gulbins, E. (1998) CD66-mediated phagocytosis of Opa52 Neisseria gonorrhoeae requires a Src-like tyrosine kinase- and Rac1-dependent signalling pathway. EMBO J 17: 443–454.

Hook, E.W., 3rd, and Holmes, K.K. (1985) Gonococcal infections. Ann Intern Med 102: 229–243.

Jerse, A.E., Sharma, N.D., Simms, A.N., Crow, E.T., Snyder, L.A., and Shafer, W.M. (2003) A gonococcal efflux pump system enhances bacterial survival in a female mouse model of genital tract infection. Infect Immun 71: 5576–5582.

© 2009 Blackwell Publishing Ltd, Cellular Microbiology, 11, 1074–1087
Johnston, R.B., Jr (2001) Clinical aspects of chronic granulomatous disease. Curr Opin Hematol 8: 17–22.

Johnson, S.R., Steiner, B.M., Cerce, D.D., Perkins, G.H., and Arko, R.J. (1993) Characterization of a catalase-deficient strain of Neisseria gonorrhoeae: evidence for the significance of catalase in the biology of N. gonorrhoeae. Infect Immun 61: 1232–1238.

Kellogg, D.S., Jr, Peacock, W.L., Jr, Deacon, W.E., Brown, L., and Pirkle, D.I. (1963) Neisseria gonorrhoeae. I. Virulence genetically linked to clonal variation. J Bacteriol 85: 1274–1279.

King, G., James, J.F., and Swanson, J. (1978) Studies on Neisseria meningitidis. J Infect Dis 137: 38–43.

Koomey, M., Gotschlich, E.C., Robbins, K., Bergstrom, S., Kulkarni, M.M., McMaster, W.R., Kamysz, E., Kamysz, W., Lee, E.H., and Shafer, W.M. (1999) The LptB, L., and Harrison, R.E., and Grinstein, S. (2003) Phagocytic killing of microbes by neutrophils. Mol Microbiol 5269–5272.

Kulkarni, M.M., McMaster, W.R., Kamysz, E., Kamysz, W., Engman, D.M., and McGwire, B.S. (2006) The major surface-metalloprotease of the parasitic protozoan, Leishmania, protects against antimicrobial peptide-induced apoptotic killing. Mol Microbiol 62: 1484–1497.

Lee, E.H., and Shafer, W.M. (1999) The farAB-encoded efflux pump mediates resistance of gonococci to long-chained antibacterial fatty acids. Mol Microbiol 33: 839–845.

Lee, W.L., Harrison, R.E., and Grinstein, S. (2003) Phagocytosis by neutrophils. Microbes Infect 5: 1299–1306.

Levy, O. (2004) Antimicrobial proteins and peptides: anti-infective molecules of mammalian leucocytes. J Leukoc Biol 76: 909–925.

Liu, G.Y., Essex, A., Buchanan, J.T., Datta, V., Hoffman, H.M., Bastian, J.F., et al. (2005) Staphylococcus aureus golden pigment impairs neutrophil killing and promotes virulence through its antioxidant activity. J Exp Med 202: 209–215.

O’Gorman, M.R., and Corrochano, V. (1995) Rapid whole-blood flow cytometry assay for diagnosis of chronic granulomatous disease. Clin Diag Lab Immunol 2: 227–232.

Ovcinnikov, N.M., and Delektorskij, V.V. (1971) Electron microscopy studies of gonococci in the urethral secretions of patients with gonorrhea. Br J Vener Dis 47: 419–439.

Qu, X.D., Harwig, S.S., Oren, A.M., Shafer, W.M., and Lehrer, R.I. (1996) Susceptibility of Neisseria gonorrhoeae to protegrins. Infect Immun 64: 1240–1245.

Ramsey, K.H., Schneider, H., Cross, A.S., Boslego, J.W., Hoover, D.L., Staley, T.L., et al. (1995) Inflammatory cytokines produced in response to experimental human gonorrhea. J Infect Dis 172: 186–191.

Rest, R.F., Fischer, S.H., Ingham, Z.Z., and Jones, J.F. (1982) Interactions of Neisseria gonorrhoeae with human neutrophils: effects of serum and gonococcal opacity on phagocyte killing and chemiluminescence. Infect Immun 36: 737–744.

Rest, R.F., and Speert, D.P. (1994) Measurement of nonopsonic phagocytic killing by human and mouse phagocytes. Meth Enzymol 236: 91–108.

Roos, D., van Bruggen, R., and Meischi, C. (2003) Oxidative killing of microbes by neutrophils. Microbes Infect 5: 1307–1315.

Schalla, W.O., Rice, R.J., Biddle, J.W., Jeanlouis, Y., Larsen, S.A., and Whittington, W.L. (1985) Lectin characterization of gonococci from an outbreak caused by penicillin-resistant Neisseria gonorrhoeae. J Clin Microbiol 22: 481–483.

Seib, K.L., Tseng, H.J., McEwan, A.G., Apicella, M.A., and Jennings, M.P. (2004) Defenses against oxidative stress in Neisseria gonorrhoeae and Neisseria meningitidis: distinctive systems for different lifestyles. J Infect Dis 190: 136–147.

Seib, K.L., Simons, M.P., Wu, H.J., McEwan, A.G., Nauseef, W.M., Apicella, M.A., and Jennings, M.P. (2005) Investigation of oxidative stress defenses of Neisseria gonorrhoeae by using a human polymorphonuclear leucocyte survival assay. Infect Immun 73: 5269–5272.

Seifert, H.S. (1997) Insertionally inactivated and inducible recA alleles for use in Neisseria. Gene 188: 215–220.

Seifert, H.S., Wright, C.J., Jerse, A.E., Cohen, M.S., and Cannon, J.G. (1994) Multiple gonococcal pilin antigenic variants are produced during experimental human infections. J Clin Invest 93: 2744–2749.

Shafer, W.M., and Rest, R.F. (1989) Interactions of gonococci with phagocytic cells. Annu Rev Microbiol 43: 121–145.

Shafer, W.M., Martin, L.E., and Spitznagel, J.K. (1986a) Late intraphagosomal hydrogen ion concentration favors the in vitro antimicrobial capacity of a 37-kilodalton cationic granule protein of human neutrophil granulocytes. Infect Immun 53: 651–665.

Shafer, W.M., Onunka, V.C., and Martin, L.E. (1986b) Antigonicoccal activity of human neutrophil cathepsin G. Infect Immun 54: 184–188.

Shafer, W.M., Qu, X., Waring, A.J., and Lehrer, R.I. (1998) Modulation of Neisseria gonorrhoeae susceptibility to vertebrate antibacterial peptides due to a member of the resistance/modulation/division efflux pump family. Proc Natl Acad Sci USA 95: 1829–1833.

Simons, M.P., Nauseef, W.M., and Apicella, M.A. (2005) Interactions of Neisseria gonorrhoeae with adherent polymorphonuclear leucocytes. Infect Immun 73: 1971–1977.

Skaar, E.P., Lazio, M.P., and Seifert, H.S. (2002) Roles of the recJ and recN genes in homologous recombination and DNA repair pathways of Neisseria gonorrhoeae. J Bacteriol 184: 919–927.

Soler-Garcia, A.A., and Jerse, A.E. (2004) A Neisseria gonorrhoeae catalase mutant is more sensitive to hydrogen peroxide and paraquat, an inducer of toxic oxygen radicals. Microb Pathog 37: 55–63.

Soler-Garcia, A.A., and Jerse, A.E. (2007) Neisseria gonorrhoeae catalase is not required for experimental genital tract infection despite the induction of a localized neutrophil response. Infect Immun 75: 2225–2233.

Spitznagel, J.K. (1990) Antibiotic proteins of human neutrophils. J Clin Invest 86: 1381–1386.

St Amant, D.C., Valentin-Bon, I.E., and Jerse, A.E. (2002) Inhibition of Neisseria gonorrhoeae by Lactobacillus species that are commonly isolated from the female genital tract. Infect Immun 70: 7169–7171.

Stohl, E.A., and Seifert, H.S. (2006) Neisseria gonorrhoeae DNA recombination and repair enzymes protect against oxidative damage caused by hydrogen peroxide. J Bacteriol 188: 7645–7651.
Stohl, E.A., Criss, A.K., and Seifert, H.S. (2005) The transcriptome response of Neisseria gonorrhoeae to hydrogen peroxide reveals genes with previously uncharacterized roles in oxidative damage protection. *Mol Microbiol* 58: 520–532.

Tobiason, D.M., and Seifert, H.S. (2006) The obligate human pathogen, Neisseria gonorrhoeae, is polyploid. *PLos Biol* 4: 1069–1078.

Virji, M., and Heckels, J.E. (1986) The effect of protein II and pili on the interaction of Neisseria gonorrhoeae with human polymorphonuclear leucocytes. *J Gen Microbiol* 132: 503–512.

WHO (2001) Global Prevalence and Incidence of Selected Curable Sexually Transmitted Infections: Overview and Estimates. Geneva: WHO.

Wu, H., Soler-Garcia, A.A., and Jerse, A.E. (2009) A strain-specific catalase mutation and mutation of the metal-binding transporter gene mntC attenuate Neisseria gonorrhoeae in vivo, but not by increasing susceptibility to oxidative killing by phagocytes. *Infect Immun* 77: 1091–1102.

**Supporting information**

Additional Supporting Information may be found in the online version of this article.

**Fig. S1.** PMNs do not require myeloperoxidase or nitric oxide synthase to kill Gc.

A. Myeloperoxidase activity is dispensable for PMNs to kill either FA1090 or ngo1686 Gc. PMNs were pretreated with 0.1 mM ABAH (dashed lines) or an equal volume of DMSO carrier (solid lines). PMNs were then infected with FA1090 Gc (diamonds) or the ngo1686 mutant (squares). The reduction in survival of ngo1686 Gc compared with the FA1090 parent in DMSO-treated PMNs is statistically significant (asterisks, *P* < 0.05, Student’s two-tailed *t*-test), but ABAH treatment had no significant effect on either FA1090 or ngo1686 survival.

B. Nitric oxide synthase activity is dispensable for PMNs to kill either FA1090 or ngo1686 Gc. PMNs from a different donor on a different day were pretreated with 0.2 mM L-NMMA (dotted lines) or an equal volume of H2O carrier (solid lines). PMNs were then infected with FA1090 Gc (diamonds) or the ngo1686 mutant (triangles). ngo1686 Gc were reduced in PMN survival compared with the FA1090 parent in H2O-treated PMNs (asterisk, *P* < 0.025, Student’s two-tailed *t*-test), but L-NMMA treatment had no significant effect on either FA1090 or ngo1686 survival.

**Table S1.** Relative association and phagocytosis of Opa– and OpaB+ FA1090 Gc with primary human PMNs.

Please note: Blackwell Publishing are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.