

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

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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 gonorrhoea. The World Health Organization estimates that 62 million cases of gonorrhoea 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 gonorrhoeal 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 Pillinger, 2001; Fichorova *et al.*, 2001; Harvey *et al.*, 2002). Although PMNs are normally capable of phagocytosing and killing a variety of microorganisms (Burg and Pillinger, 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

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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 carcinoembryonic 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 (Fauschou 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).

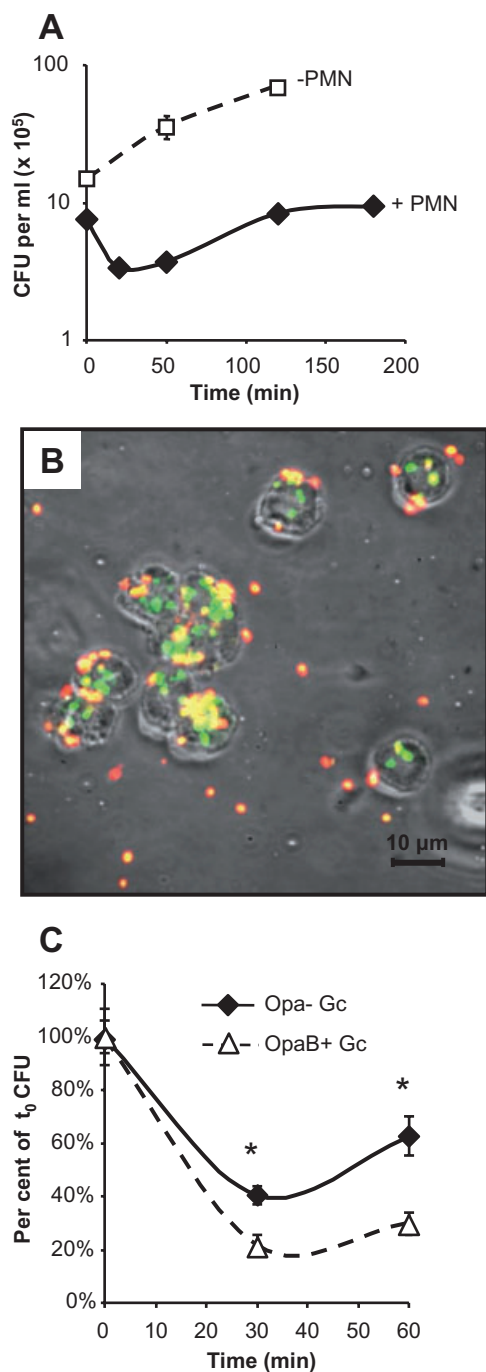
In this study we examined Gc survival after exposure to attached, IL-8-primed PMNs as a model for phagocytes that have migrated to the inflamed urogenital tract. We found that a sizable percentage of the bacterial inoculum survived early exposure to PMNs and both intracellular and extracellular Gc remained 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

Fig. 1. Adherent, IL-8-primed PMNs phagocytose and kill a subset of piliated Gc.

A. Reduced survival of Gc after PMN infection. Piliated Gc of strain FA1090 were exposed to adherent, IL-8-primed PMNs at a multiplicity of infection of 1 (solid line), or were maintained in infection medium without PMNs (dashed line), and the average cfu \pm SEM recovered over 180 min were determined by serial dilution and plate count. Among different experiments, Opa- Gc survival over the first 60 min of infection ranged from 40% to 70% of the cfu present at t_0 . As each PMN preparation had a different level of bactericidal activity against Gc, the results shown are from a single experiment but are representative of multiple experiments conducted with PMNs from different donors.

B. PMNs phagocytose unopsonized Opa- Gc. PMNs were infected with Gc at a multiplicity of infection of 8 for 30 min, and intracellular and extracellular Gc were discriminated from one another based on accessibility to a Gc-specific antibody before and after PMN permeabilization. Extracellular Gc 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 \pm 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 (t_0). Asterisk, $P < 0.01$ between Opa- and OpaB+ Gc at matched time points (Student's two-tailed *t*-test).

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 yellow (red + green fluorescence), while intracellular bacteria, which were not accessible to the red fluorescent secondary antibody, appear green. PMNs were infected with Opa- Gc at a higher multiplicity of infection (~ 8 cfu per PMN) to maximize bacteria-PMN interactions for imaging. Images were captured by confocal laser scanning microscopy of a single optical slice with an approximate thickness of ~ 130 nm. Of the 57 PMNs examined after 30 min of infection, 55 had associated Gc. Each infected PMN was associated with an average of 10 fluorescent bacterial particles, correlating well with the number of added bacteria by cfu calculation. Forty-seven per cent of the bacterial particles associated with PMNs were intracellular at this time point

(Fig. 1B). 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

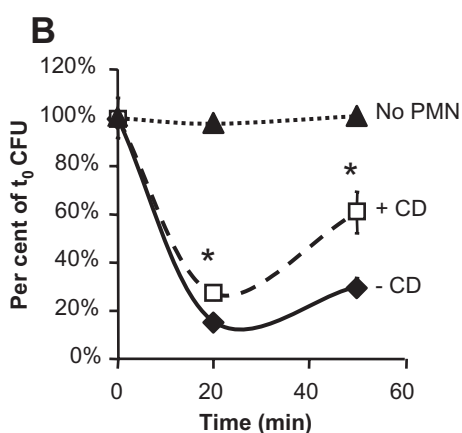
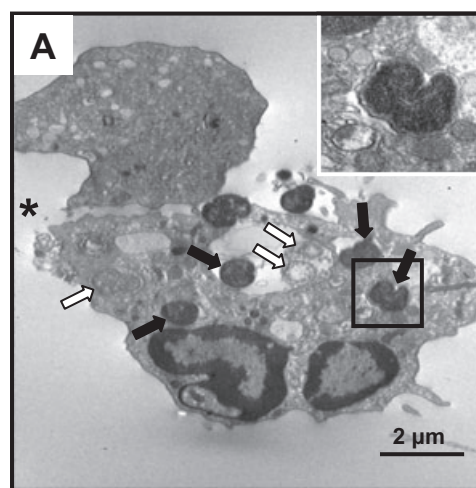


Fig. 2. PMNs have antigonococcal activity against intracellular and extracellular bacteria.

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).

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

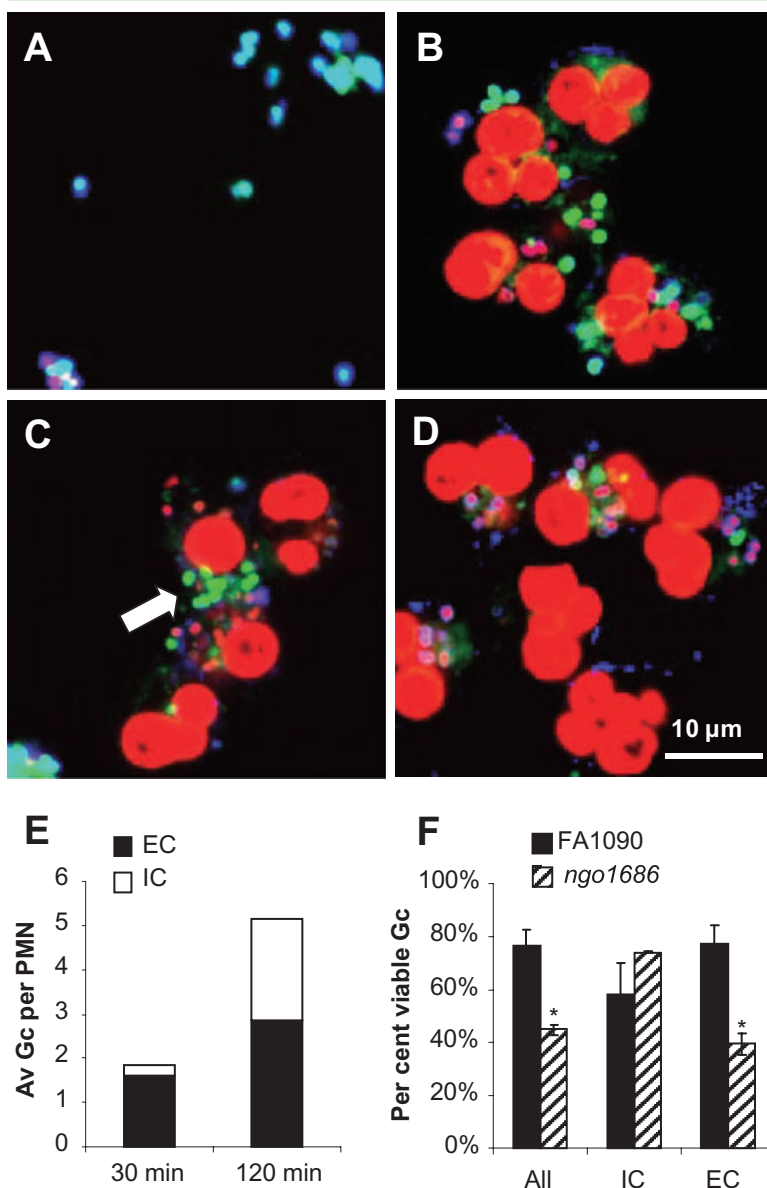


Fig. 3. Direct viability staining of Gc during PMN infection reveals that PMNs can kill extracellular Gc and a subset of intracellular Gc survive PMN challenge. FA1090 Gc were cultured in infection medium without PMNs for 30 min (A), or used to infect adherent, IL-8-treated PMNs for 30 min (B) or 120 min (C). In (D), PMNs were infected with *ngo1686* Gc for 30 min. Gc and Gc-infected PMNs were exposed to SBA (blue) to selectively identify extracellular bacteria, followed by saponin permeabilization and exposure to the BacLight viability dyes SYTO9 (green) and PI (red), which stain viable and non-viable bacteria respectively. Viable intracellular bacteria appear green, viable extracellular bacteria appear turquoise blue, non-viable internalized bacteria appear red and non-viable extracellular bacteria appear magenta. Arrow in (C) indicates a cluster of viable, intracellular Gc, potentially indicative of bacterial replication inside PMNs. (E) is a graphical representation of the images in (B) and (C), showing the average number of viable FA1090 Gc located intracellularly (IC) or extracellularly (EC) to PMNs after 30 or 120 min of infection. (F) is a graphical representation of the images in (B) and (D), showing the percentage of viable parental FA1090 and *ngo1686* mutant bacteria associated with PMNs as follows: All, both intracellular and extracellular bacteria; IC, intracellular bacteria; EC, extracellular bacteria. The difference between the two strains for all viable bacteria and the extracellular population is significant (asterisk, $P < 0.025$, Student's two-tailed *t*-test) but is not significantly different for the intracellular population.

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⁺, 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 PMNs, 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 gonorrhoeal 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 (Criss 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 diphenylidene iodonium 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

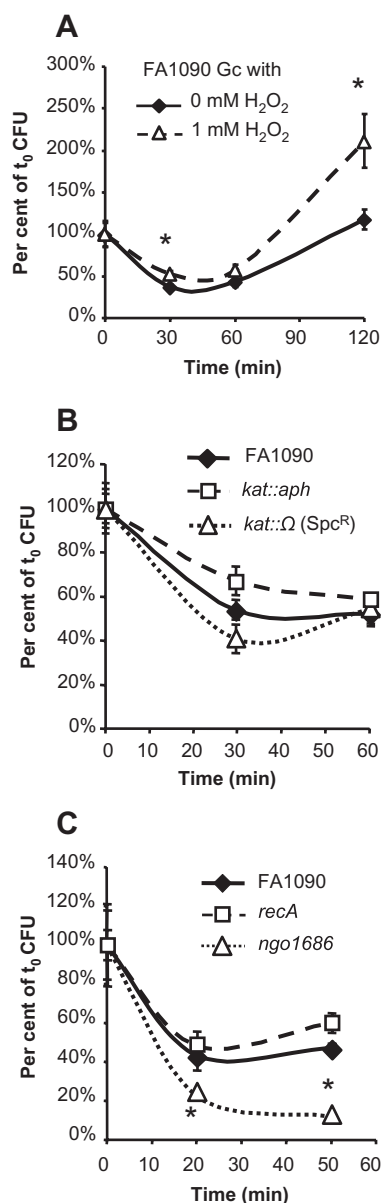


Fig. 4. Gc with diminished antioxidant defences vary in their survival to PMNs.

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^R], 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).

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 anticonococcal 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 anticonococcal 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

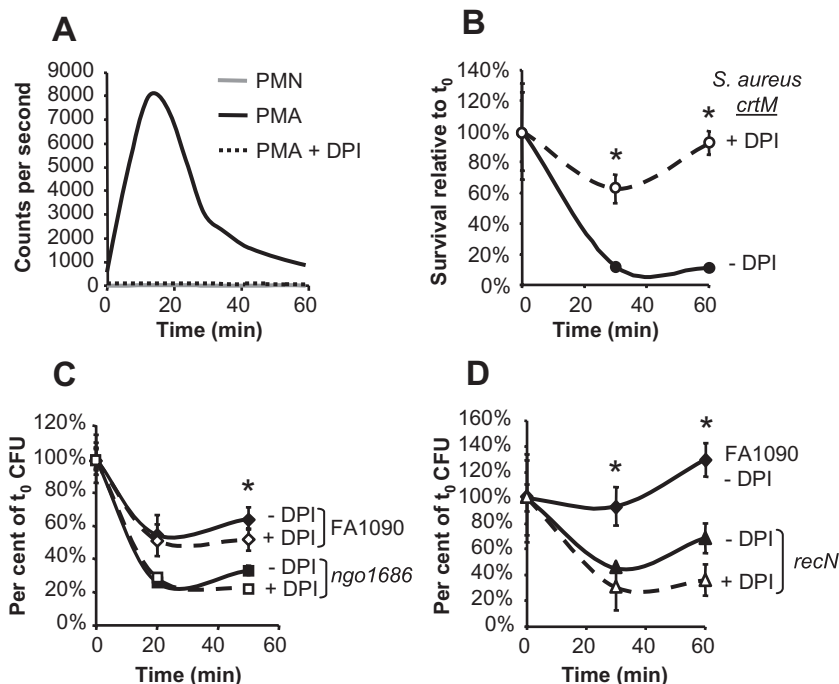


Fig. 5. Treatment of PMNs with the NADPH oxidase inhibitor DPI does not affect Gc survival.

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 PMNs than the *ngo1686* mutant (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.

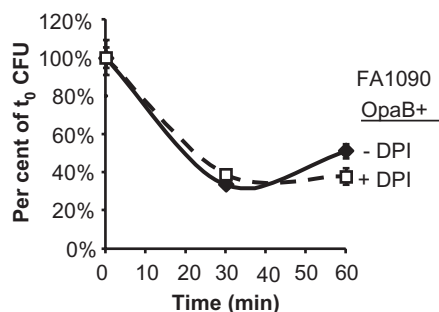


Fig. 6. DPI treatment does not enhance the survival of OpaB+ Gc in PMNs. PMNs were pretreated with DPI (dashed line) or DMSO (solid line), then infected with FA1090 Gc expressing the CEACAM-binding OpaB. DPI treatment had no significant effect on the survival of OpaB+ Gc.

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 gonorrhoeal 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

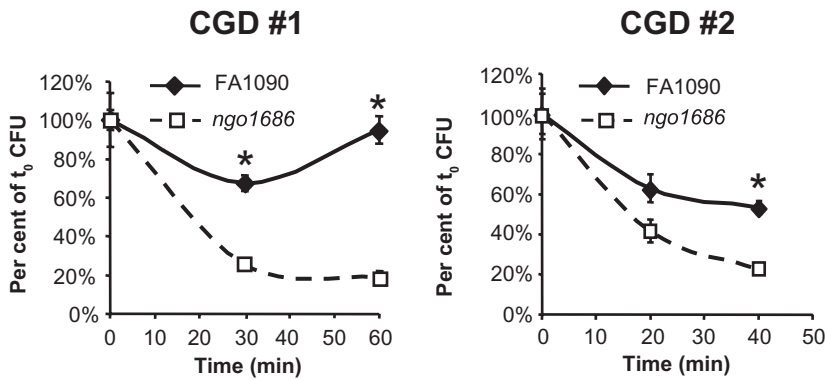


Fig. 7. PMNs from CGD patients, genetically deficient in NADPH oxidase activity, retain the ability to differentially kill parental and *ngo1686* Gc. PMNs were isolated from two unrelated individuals with CGD (CGD #1 and CGD #2) and infected *in vitro* as in Fig. 1A with FA1090 Gc (solid line) or the *ngo1686* mutant (dashed line). The *ngo1686* mutant survived significantly less well than the parental strain in CGD PMNs (asterisks, $P < 0.05$ for FA1090 versus *ngo1686* Gc at matched time points, Student's two-tailed *t*-test).

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 phenotypic 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 phagolysosomes, 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 antigonococcal 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

(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::tetM*, *ngo1686::ermC*, *recN::ermC* and *kat::aph* mutants and the OpaB+ 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::Ω(spc^R)* mutant was obtained from S. Johnson (United States Centers for Disease Control) (Johnson *et al.*, 1993). The *kat::Ω* mutation was introduced into FA1090 1-81-S2 Gc by natural transformation, colonies resistant to 40 µg ml⁻¹ spectinomycin were isolated, and replacement of the parental *kat* allele with *kat::Ω* 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 cytochrome 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 1×10^7 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 N^G-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 pre-treatment 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 as unbiased a 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 as unbiased a 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 Thermanox 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).

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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.

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