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Neisseria gonorrhoeae suppresses the oxidative burst of human polymorphonuclear leukocytes

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Summary

Symptomatic infection with *Neisseria gonorrhoeae* (Gc) results in a potent polymorphonuclear leukocyte (PMN)-driven inflammatory response, but the mechanisms by which Gc withstands PMN attack are poorly defined. Here we report that Gc can suppress the PMN oxidative burst, a central component of the PMN antimicrobial arsenal. Primary human PMNs remained viable after exposure to liquid-grown, exponential-phase, opacity-associated protein (Opa)-negative Gc of strains FA1090 and MS11 but did not generate reactive oxygen species (ROS), even after bacterial opsonization. Liquid-grown FA1090 Gc expressing OpaB, an Opa protein previously correlated with PMN ROS production, elicited a minor PMN oxidative burst. PMN ROS production in response to Opa$^-$ and OpaB$^+$ Gc was markedly enhanced if bacteria were agar-grown or if liquid-grown bacteria were heat-killed. Liquid-grown Opa$^-$ Gc inhibited the PMN oxidative burst elicited by isogenic dead bacteria, formylated peptides or *Staphylococcus aureus* but did not inhibit PMN ROS production by OpaB$^+$ Gc or phorbol esters. Suppression of the oxidative burst required Gc–PMN contact and bacterial protein synthesis but not phagocytosis. These results suggest that viable Gc directly inhibits PMN signalling pathways required for induction of the oxidative burst, which may contribute to gonococcal pathogenesis during inflammatory stages of gonorrhoeal disease.

Introduction

The obligate human pathogen *Neisseria gonorrhoeae* (the gonococcus; Gc) is the causative agent of the sexually transmitted infection gonorrhoea, which affects over 62 million individuals worldwide every year (WHO, 2001). Acute gonococcal infection elicits a potent inflammatory response characterized primarily by the influx of polymorphonuclear leukocytes (neutrophils or PMNs) to the lower urogenital tract (Harkness, 1948). The resulting exudate consisting of PMNs with attached and internalized Gc is the clinical hallmark of gonorrhoeal disease (Shafer and Rest, 1989). Although PMNs possess a broad range of antimicrobial activities, Gc can be cultured from gonorrhoeal exudates, demonstrating that the bacteria can survive the host inflammatory response (Hook and Holmes, 1985). Gc defence against PMN killing undoubtedly contributes to the continued persistence of gonorrhoea within the human population.

Indirect evidence suggests that Gc survive exposure to PMNs by defending against phagocyte-derived reactive oxygen species (ROS). In activated PMNs, the NADPH oxidase enzyme converts molecular oxygen into superoxide (reviewed in Roos *et al.*, 2003). Superoxide serves as the precursor for other ROS including hydrogen peroxide and hypochlorous acid or bleach, which is generated by the PMN granule enzyme myeloperoxidase (Hampton *et al.*, 1998). ROS have potent antimicrobial activity *in vitro* and additionally facilitate the release of PMN granule enzymes and peptides that are toxic for many microorganisms (Fang, 2004; Segal, 2005). The Gc genome encodes an extensive array of antioxidant gene products, many of which have been shown to protect the organism from ROS-mediated killing. Some of these gene products directly detoxify ROS (e.g. catalase, superoxide dismutase, cytochrome *c* peroxidase and a manganese-dependent ROS quenching system), while others protect Gc from ROS-mediated damage to biomolecules (e.g. peptide methionine sulfoxide reductase and RecN) (reviewed in Seib *et al.*, 2006). Our laboratory has recently shown that expression of many of these gene products is upregulated in Gc exposed to sublethal concentrations of hydrogen peroxide, and a subset of ROS-induced genes protect the bacteria from ROS and PMN-mediated killing (Stohl *et al.*, 2005). The retention of this array of gene products in the Gc genome and the observation that their expression is responsive to oxidative stress imply that Gc encounter ROS as part of their life cycle and are adept at correcting the resulting damage.

Other evidence suggests that PMNs use means that are independent of ROS production to combat Gc infection. Seib *et al.* found that inactivation of Gc

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antioxidant genes, singly or in combination, had no effect on bacterial survival after exposure to PMNs, and a Gc catalase mutant was not compromised for survival in the murine genital tract (Seib *et al*., 2005; Soler-Garcia and Jerse, 2007). Furthermore, PMNs maintained in anoxic conditions or from patients with chronic granulomatous disease (CGD), who are genetically deficient in NADPH oxidase, retain some antigonococcal activity (Rest *et al*., 1982; Frangipane and Rest, 1992). PMNs may therefore combat Gc infection with antimicrobial factors that function independently of ROS. Non-oxidative PMN antimicrobial factors that reduce Gc viability *in vitro* include cathepsin G and the cationic antimicrobial peptide LL-37 (Rest and Pretzer, 1981; Shafer *et al*., 1986; 1998; Qu *et al*., 1996), which are resisted in part by the activity of the Gc Mtr efflux pump (Shafer *et al*., 1998). The Mtr system facilitates Gc survival in the murine genital tract (Jerse *et al*., 2003; Warner *et al*., 2007), but whether Mtr directly protects Gc from PMN killing is not known. Thus, the non-oxidative factors directed against Gc *in vivo* and the Gc gene products that confer protection from these factors still remain to be defined.

Central to the question of whether PMNs direct oxidative or non-oxidative antimicrobial mechanisms against Gc is the degree to which PMNs generate ROS during gonorrhoeal infection. The reported ability of PMNs to mount an oxidative burst in response to Gc varies widely, with some of the variation due to differences in bacterial outer membrane surface components. ROS production in PMNs is stimulated by Gc that express selected members of the opacity-associated (Opa) protein family, which engage carcinoembryonic antigen-cell adhesion molecule (CEACAM) receptors on the phagocyte surface and facilitate bacterial internalization (Dehio *et al*., 1998). Opa expression alone is sufficient to promote ROS production in PMNs, as shown using *Escherichia coli* heterologously expressing certain CEACAM-binding Opa proteins (Belland *et al*., 1992; Chen and Gotschlich, 1996). However, the Opa-mediated PMN oxidative burst is less potent than stimuli such as opsonized zymosan (Simons *et al*., 2005). In the absence of Opa expression, Gc fail to stimulate a sizable PMN oxidative burst, but the strains tested in these studies also lacked adhesins (e.g. type IV pili) that could potentially facilitate Opa-independent interactions with PMNs (Rest *et al*., 1982; Virji and Heckels, 1986; Fischer and Rest, 1988; Elkins and Rest, 1990). In re-examining the data in these reports, Opa$^-$ Gc do appear to trigger some degree of ROS production from PMNs, albeit less than their Opa$^+$ counterparts (Rest *et al*., 1982; Virji and Heckels, 1986). Given these discrepancies in the literature, it is apparent that key questions remain regarding PMN responsiveness to Gc infection.

Here we have directly monitored the oxidative burst in primary human PMNs exposed to different strains of liquid-grown, exponential-phase Gc. Strikingly, piliated, Opa$^-$ Gc not only failed to elicit ROS production in PMNs but also suppressed the oxidative burst in response to a subset of factors known to stimulate NADPH oxidase activity. Suppression required live bacteria and occurred without affecting PMN viability, suggesting that Gc use an active process to prevent PMN ROS production. These results identify a previously unappreciated mechanism by which Gc subvert PMN antimicrobial activities, which may assist the organism in establishing and maintaining infection in its obligate human host.

Results

PMNs do not produce a significant oxidative burst in response to live, exponentially growing Gc

The ability of primary human PMNs to generate ROS following infection with live Gc was monitored using luminol-dependent chemiluminescence (LDCL) (Dahlgren and Karlsson, 1999). PMNs were isolated from venous blood of healthy human donors and incubated with 20 μM luminol in a defined glucose-rich medium that supports survival of PMNs and growth of Gc (Morse and Bartenstein, 1980). ROS production was measured as counts per second of luminescence at 5 min intervals over 1 h. Unstimulated PMNs did not produce detectable ROS, whereas PMN exposure to the phorbol ester phorbol myristate acetate (PMA) potently stimulated ROS production within 15 min (Fig. 1A). PMA-elicited LDCL was completely abolished in the presence of the NADPH oxidase inhibitor diphenylidene iodonium hydrochloride, showing that PMN ROS production in this system required NADPH oxidase activity (data not shown). The magnitude of the oxidative burst of primary PMNs to PMA was found to vary among experiments, with variation found between donors, as well as from each individual donor on different days. Thus, comparisons between strains or treatment conditions were made within a single experiment conducted on one day with one donor's cells, and all results shown are representative of several experiments conducted with PMNs from at least three different donors.

LDCL was then monitored in PMNs infected with Gc of strains FA1090 and MS11. For these experiments, piliated, Opa$^-$ Gc were grown in liquid culture for multiple rounds of replication; this protocol enriches for live, actively growing bacteria and has been previously used in our laboratory to demonstrate that the majority of Gc survive 1 h exposure to PMNs, which is aided by the bacterial gene products RecN and Ngo1686 (Stohl *et al*., 2005). PMNs exposed to FA1090 and MS11 Gc at multiplicities of infection (moi) of ~100 colony-forming units (cfu) per PMN generated only 4% and 2% of the LDCL elicited by PMA stimulation respectively (Fig. 1A). No oxidative burst was detected in

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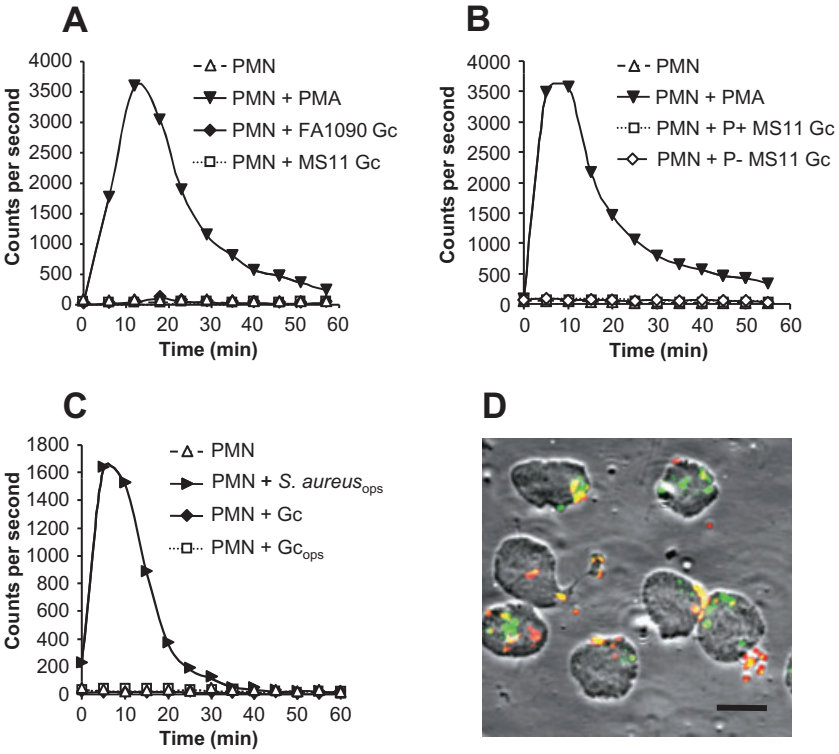

Fig. 1. Viable, exponentially growing Opa⁻ Gc do not induce the oxidative burst in PMNs.
A. Absence of the oxidative burst in Gc-infected PMNs. Primary human PMNs were left uninfected, stimulated with PMA, or exposed to piliated, Opa⁻ Gc of strains FA1090 (moi 90) or MS11 (moi 80). ROS production was measured as counts per second of LDCL.
B. Neither piliated nor non-piliated Gc stimulate PMN oxidative metabolism. ROS production was measured in PMNs infected with MS11 Gc that were piliated (p^+; moi 100) or constitutively non-piliated due to deletion of the *pilE* pilin structural gene (p^-; moi 94).
C. Neither opsonized nor unopsonized Gc stimulate PMN oxidative metabolism. ROS production was measured in PMNs infected with unopsonized MS11 Gc (moi 200) or bacteria opsonized in 10% autologous serum, either MS11 Gc (Gc_{ops}; moi 130) or *S. aureus* (*S. aureus*$_{ops}$; moi 40).
D. PMNs bind and phagocytose unopsonized Gc. PMNs were infected with unopsonized FA1090 Gc at an moi of 10 for 30 min. Intracellular and extracellular bacteria were discriminated from one another based on accessibility to a Gc-specific antibody before and after PMN permeabilization. Extracellular bacteria appear red/yellow; intracellular bacteria appear green. Scale bar = 10 μm.

PMNs exposed to moi of Gc ranging from 25 to 500 cfu per PMN or when infection was continued for > 2 h (data not shown). The absence of a notable oxidative burst was not attributable to the use of piliated Gc, as no ROS production was observed in PMNs exposed to isogenic non-piliated bacteria (Fig. 1B). Neither unopsonized Gc nor bacteria opsonized with autologous human serum elicited the oxidative burst in PMNs (Fig. 1C). The normal human serum retained opsonizing activity, as *Staphylococcus aureus* incubated with serum under the same conditions promoted strong ROS production from PMNs (Fig. 1C), whereas unopsonized *S. aureus* did not (data not shown). Gc-infected PMNs also did not generate detectable ROS by lucigenin- or isoluminol-enhanced chemiluminescence (data not shown). Therefore, PMNs fail to mount a respiratory burst when presented with either of two unrelated strains of viable, exponentially growing Gc.

We tested and discounted several possibilities that could explain the absence of a sizable oxidative burst in PMNs exposed to Gc. The lack of ROS production was not due to inefficient bacteria–host cell contact, because no oxidative burst was detected even after Gc were centrifuged onto PMN monolayers (data not shown), which enhanced bacterial attachment and phagocytosis (Fig. 1D). Nor was it due to increased PMN lysis, as the amount of lactate dehydrogenase (LDH) activity released into PMN supernatants was not affected by Gc infection; in contrast, *Pseudomonas aeruginosa* secreting the cytotoxin ExoU stimulated significantly greater release of LDH from PMNs (Fig. 2A). Moreover, neither uninfected PMNs nor PMNs exposed to liquid-grown Gc exhibited morphological changes characteristic of apoptosis during the time-course of the assay, indicating that Gc infection did not promote either necrotic or apoptotic PMN death (data not shown). Finally, Gc catalase activity was not detoxifying PMN-derived ROS such that it could not be detected by LDCL, because catalase-deficient Gc also failed to promote ROS production in PMNs (Fig. 2B). These results show that PMNs phagocytose Gc and that interaction with Gc does not compromise PMN viability, suggesting that Gc may be directly preventing PMNs from mounting the oxidative burst.

Previous reports have indicated that PMNs mount a strong oxidative burst in response to Gc Opa proteins that engage granulocyte CEACAM receptors, such as OpaB in strain FA1090 (Elkins and Rest, 1990; Naids and Rest, 1991; Dehio *et al.*, 1998). In contrast, we observed that live, exponentially growing, piliated FA1090 Gc that predominantly expressed OpaB ($OpaB^+$) elicited minimal ROS production from PMNs, only slightly greater than the isogenic Opa⁻ parent (Fig. 3A). While Opa⁻ Gc elicited essentially no oxidative burst in PMNs (total LDCL was < 1% of that stimulated by PMA), LDCL in response to $OpaB^+$ Gc was 5% of the PMA-induced burst. Similar results were obtained with a FA1090 Gc parent carrying deletions of all 11 *opa* genes and an isogenic strain in which *opaB* was re-introduced at a second location in the Gc chromosome (Fulcher, 2004; data not shown). The effect of

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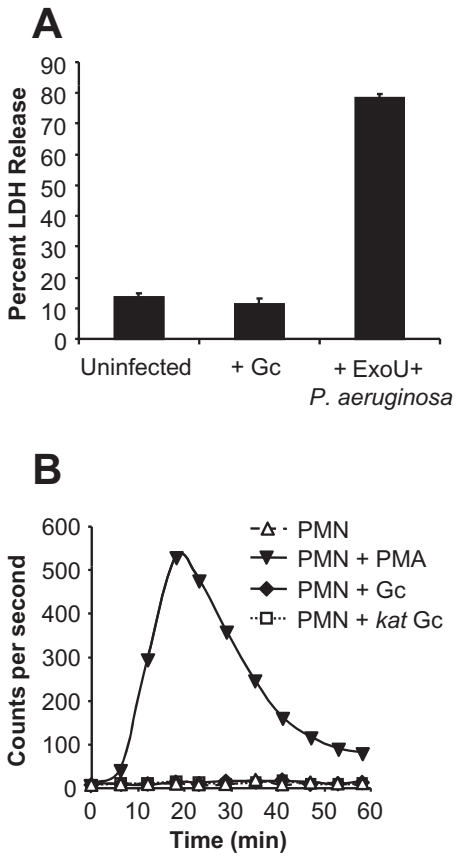

Fig. 2. The absence of an oxidative burst in Gc-infected PMNs is not due to reduced PMN viability or catalase-mediated degradation of PMN-derived ROS.
A. PMNs remain viable after infection with Gc at high moi. PMNs were left uninfected or were infected with MS11 Gc (moi 390) for 1 h. Lactate dehydrogenase (LDH) release into the supernatant is expressed as a percentage of total cellular LDH activity. PMNs infected with ExoU-expressing *P. aeruginosa* for 3 h served as a positive control for PMN lysis.
B. No detectable PMN oxidative burst in PMNs infected with catalase-deficient Gc. PMNs were left uninfected, stimulated with PMA, or infected with FA1090 Gc (moi 80) or an isogenic *kat* mutant (moi 50). ROS production was measured as in Fig. 1A.

OpaB$^+$ Gc on the PMN oxidative burst was independent of bacterial piliation state, because OpaB$^+$ Gc carrying a deletion in *pilE* elicited similar amounts of ROS from PMNs as their isogenic piliated counterparts (data not shown). The OpaB$^+$-stimulated oxidative burst required bacterial phagocytosis, as ROS production was abolished in PMNs treated with cytochalasin D (CD) (data not shown). These results show the respiratory burst elicited from PMNs by liquid-grown OpaB$^+$ Gc is minimal compared with the effect of other soluble (PMA) or particulate (*S. aureus*) stimuli.

Nonviable Gc stimulate ROS production in PMNs

The weak ROS response of PMNs to liquid-grown OpaB$^+$ Gc led us to consider whether bacterial growth conditions influenced the ability of PMNs to mount an oxidative burst. Previous work examining Opa$^+$ Gc interactions with PMNs typically used bacteria collected from agar-containing medium or grown in liquid culture for short times (< 3 h) prior to infection (Rest *et al.*, 1982; Virji and

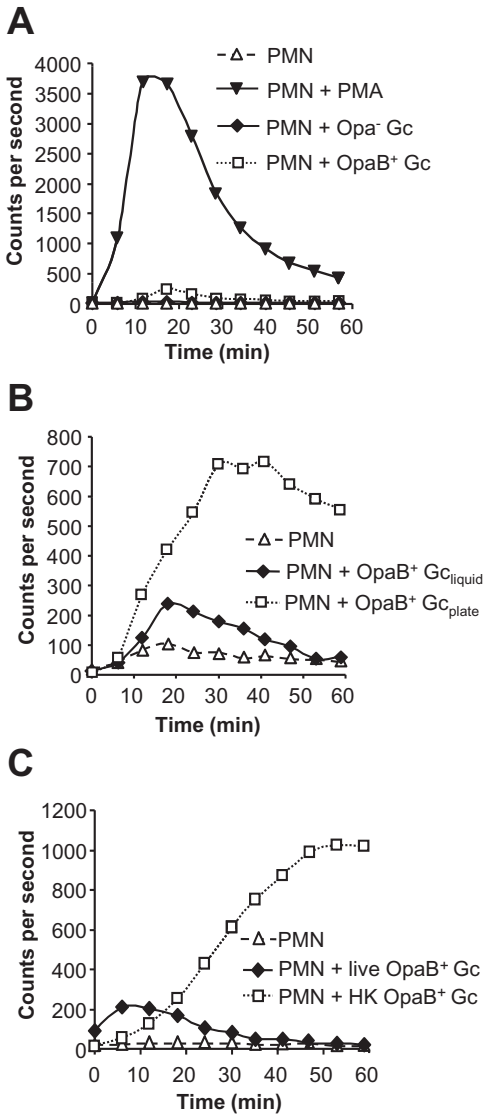

Fig. 3. Live, Opa-expressing Gc elicit a minimal oxidative burst in PMNs.
A. Minor oxidative burst in PMNs infected with live, exponentially growing OpaB$^+$ FA1090 Gc. PMNs were left uninfected, stimulated with PMA, or exposed to liquid-grown, exponential-phase FA1090 Gc that were Opa$^-$ (moi 82) or expressed OpaB (OpaB$^+$ moi 120). ROS production was measured as in Fig. 1A.
B. Enhanced ROS production in PMNs infected with agar-grown OpaB$^+$ Gc. ROS production was measured in PMNs that were left uninfected or infected with OpaB$^+$ Gc that were grown in liquid medium to exponential phase (Gc_{liquid}; moi 160) or on agar-containing medium (Gc_{plate}; moi 14).
C. Enhanced ROS production in PMNs exposed to liquid-grown, heat-killed OpaB$^+$ Gc. ROS production was measured in PMNs that were left uninfected, or infected with moi 260 of liquid-grown OpaB$^+$ Gc that were live or had been killed by heating (HK).

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Heckels, 1986; Fischer and Rest, 1988; Naids and Rest, 1991; Gray-Owen *et al*., 1997; Simons *et al*., 2005). When FA1090 OpaB$^+$ Gc were harvested from agar plates and presented to PMNs, they elicited eightfold more total LDCL from PMNs than liquid-grown Gc, even though the viable cfu present in the liquid-grown culture outnumbered the plate-grown by a factor of 10 (Fig. 3B). ROS production in response to plate-grown bacteria was first detected after ~15 min and reached a maximum after 30–40 min, persisting at that level for > 60 min of total exposure (Fig. 3B). We reasoned that the increased responsiveness of PMNs to plate-grown OpaB$^+$ Gc could be due either to changes in bacterial physiology under different growth conditions or to dead bacteria and bacterial products that are more abundant in plate-grown preparations than in liquid culture. To test the latter possibility, live and dead Opa-expressing Gc were compared in their ability to elicit PMN ROS production. Liquid-grown OpaB$^+$ FA1090 Gc were heated to 56°C for 30 min, a condition that inhibits autolysis (Hebeler and Young, 1975; Elmros *et al*., 1976) and results in a complete loss of bacterial viability (data not shown). The heat-killed Gc elicited 12-fold more total ROS production in PMNs than their live counterparts (Fig. 3C), and ROS production in PMNs exposed to heat-killed and plate-grown OpaB$^+$ Gc had similar kinetics (compare Fig. 3B and C). These results show that the minimal oxidative burst in PMNs exposed to liquid-grown, exponential-phase OpaB$^+$ Gc can be enhanced by intact, dead bacteria as well as bacterial factors present in agar-grown Gc preparations.

To test whether the stimulation of the PMN oxidative burst by non-viable Gc could also be observed with Opa$^-$ bacteria, liquid-grown Opa$^-$ Gc were killed by heating. Heat-killed Opa$^-$ MS11 Gc stimulated PMN ROS production, while an equal number of live bacteria did not: heat-killed Opa$^-$ Gc elicited 39 times greater total ROS produced from PMNs than viable Gc (Fig. 4A). Similar results were obtained for Opa$^-$ FA1090 Gc (data not shown). The kinetics of ROS production in PMNs stimulated with Opa$^-$ and OpaB$^+$ bacteria were similar (compare Figs 3B and 4A). Opa$^-$ bacteria killed by isopropanol treatment also promoted PMN ROS production, indicating that the stimulatory property of non-viable Gc on PMNs was not specifically released by heating (data not shown). Taken together, these results demonstrate that dead Gc, regardless of their Opa profile, retain the ability to induce the oxidative burst in PMNs, but live bacteria can overcome this effect.

Based upon these observations, we hypothesized that Gc had the ability to stimulate ROS metabolism in PMNs, but liquid-grown, exponential-phase Gc made additional products that prevented PMNs from producing ROS. To test whether bacterial protein synthesis was necessary for viable Gc to prevent the PMN oxidative burst, FA1090 Gc were pre-treated with chloramphenicol or tetracycline at concentrations previously determined for this strain not to affect bacterial viability but sufficient to prevent initiation of new rounds of replication (Tobiason and Seifert, 2006). The antibiotic-treated Gc did not exhibit any survival defect (data not shown) but elicited a strong oxidative burst in PMNs compared with untreated bacteria (Fig. 4B). Antibiotic-treated Gc stimulated the release of ROS from PMNs with similar kinetics and potency to heat-killed bacteria, but ROS production did not persist in response to antibiotic-treated Gc as it did for heat-killed bacteria, for reasons that remain obscure (Fig. 4B). This result shows that liquid-grown, exponential-phase Gc prevent PMNs from producing ROS by a process that requires *de novo* bacterial protein synthesis. These results lead us to conclude that viable Gc undergoing protein synthesis actively prevent PMNs from mounting the oxidative burst.

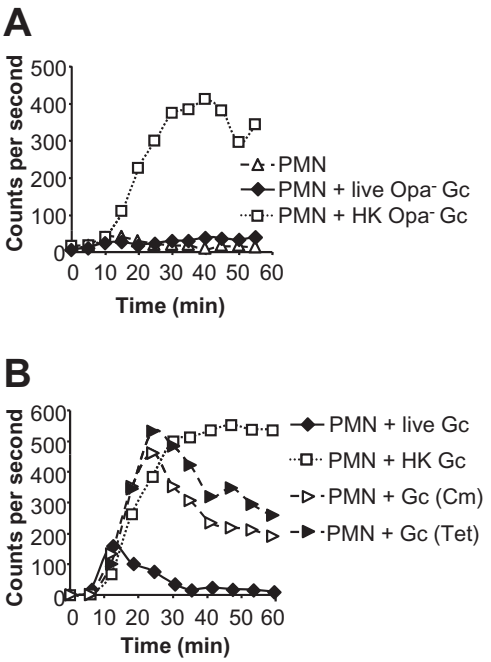

Fig. 4. The absence of substantial ROS production in PMNs exposed to liquid-grown, exponential-phase Gc requires live bacteria capable of *de novo* protein synthesis.
A. Liquid-grown, heat-killed Opa$^-$ Gc stimulate ROS production in PMNs. PMNs were left uninfected or were infected with liquid-grown Opa$^-$ MS11 Gc that were live (moi 120) or killed by heating at 56°C for 30 min (HK; concentration equivalent to moi 250). ROS production was measured as in Fig. 1A.
B. Viable Opa$^-$ Gc treated with antibiotics that inhibit protein synthesis stimulate PMN ROS production. PMNs were infected with liquid-grown Opa$^-$ FA1090 Gc at an moi of 100. Gc were exponentially growing (live), heat-killed (HK), or treated with sublethal concentrations of the protein synthesis inhibitors chloramphenicol (Cm) or tetracycline (Tet). Uninfected PMNs did not generate any detectable ROS (data not shown).

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Live Gc suppress the PMN oxidative burst

If viable Gc actively block PMN ROS production, they should not only fail to induce the oxidative burst on their own but also should inhibit the oxidative burst stimulated by dead bacteria. To directly test this hypothesis, PMNs were exposed to liquid-grown, exponential-phase, Opa⁻ Gc, isogenic heat-killed bacteria, or a combination of the two. Live MS11 Gc failed to elicit ROS production in PMNs at an moi of either 200 or 500 (Fig. 5A; only the results for moi 500 are presented), while heat-killed bacteria stimulated PMN oxidative metabolism in an moi-dependent manner. When equivalent numbers of viable and heat-killed Gc were added to PMNs, the PMN oxidative burst was completely abrogated (Fig. 5A). Live Gc retained the ability to inhibit the PMN oxidative burst elicited by heat-killed Gc when outnumbered 2.5-fold by dead bacteria, reducing the burst to 60% of that elicited by heat-killed Gc alone (Fig. 5A). We conclude that live Opa⁻ Gc actively inhibit the PMN respiratory burst elicited by non-viable bacteria.

To examine whether the ability of live Gc to inhibit the PMN oxidative burst could be observed for other stimuli, LDCL was monitored in PMNs infected with live Opa⁻ Gc and exposed to factors known to elicit PMN ROS production. PMNs stimulated with 10 μM formylmethionylleucylphenylalanine (fMLP) generated ROS that were detectable after 5–10 min, reached a maximum after 30 min, and declined to basal levels after 60 min (Fig. 5B). Infection with Opa⁻ FA1090 or MS11 Gc reduced the fMLP-induced oxidative burst by 60% (Fig. 5B and data not shown). This reduction was not due to bacterial sequestration or degradation of the peptide, as fMLP solution that was pre-incubated with Gc elicited the same amount of ROS production from PMNs as fresh fMLP (data not shown). Gc also affected the PMN oxidative burst elicited by serum-opsonized *S. aureus*. At an moi of 100, *S. aureus* promoted ROS production in PMNs that peaked after 20 min and declined to basal levels by 35 min (Fig. 5C). Co-infection of PMNs with equivalent numbers of *S. aureus* and Gc reduced the *S. aureus*-induced oxidative burst by 84% (Fig. 5C). In experiments where Gc outnumbered *S. aureus* by fivefold, the *S. aureus*-induced burst was almost completely abolished, indicating that the inhibitory effect of Gc was dose-dependent (data not shown). Notably, the suppressive effect of Gc on both the fMLP- and *S. aureus*-induced PMN oxidative burst required viable, Opa⁻ bacteria, as heat-killed or OpaB⁺ Gc enhanced the burst elicited by these stimuli (data not shown).

To test whether the suppression of PMN oxidative metabolism extended to all stimulatory factors, the oxidative burst was measured in PMNs infected with Gc and stimulated with PMA, which directly activates protein kinase C isoforms necessary for NADPH oxidase activation (Nauseef *et al.*, 1991). Viable Opa⁻ Gc did not affect the magnitude of the oxidative burst elicited by PMA, even when PMNs were incubated with Gc for 15 min prior to PMA addition, whereas Gc inhibited the *S. aureus*-

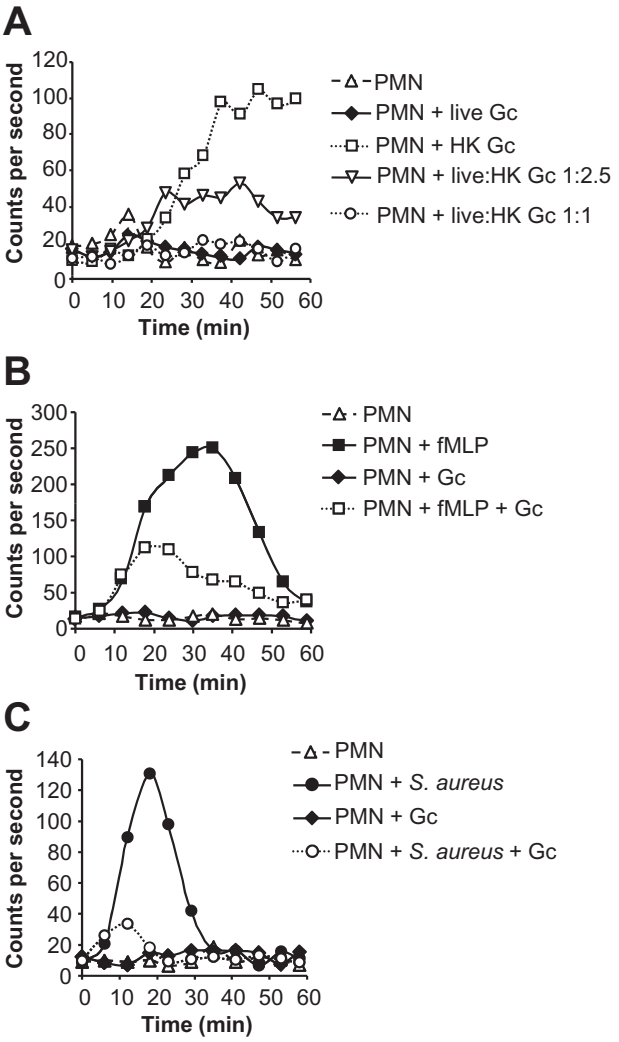

Fig. 5. Live Opa⁻ Gc suppress the oxidative burst of PMNs stimulated with isogenic heat-killed Gc, formylated peptides and *S. aureus*.
A. Live Opa⁻ Gc suppress PMN ROS production elicited by heat-killed bacteria. ROS production was measured in PMNs that were left uninfected, exposed to liquid-grown Opa⁻ MS11 Gc that were live (moi 500) or heat-killed (HK; concentration equivalent to moi 500), or mixtures of live and heat-killed bacteria at the indicated ratios. ROS production was measured as in Fig. 1A. Viable Gc also failed to elicit ROS production in PMNs at an moi of 200 (data not shown).
B. Gc suppress the fMLP-stimulated PMN oxidative burst. PMNs were left untreated or were exposed to 10 μM fMLP, MS11 Gc at an moi of 100, or fMLP and Gc simultaneously.
C. Gc suppress the PMN oxidative burst elicited by opsonized *S. aureus*. PMNs were left untreated or were exposed to serum-opsonized *S. aureus* (moi 100), FA1090 Gc (moi 100), or *S. aureus* and Gc simultaneously (each at moi 100).

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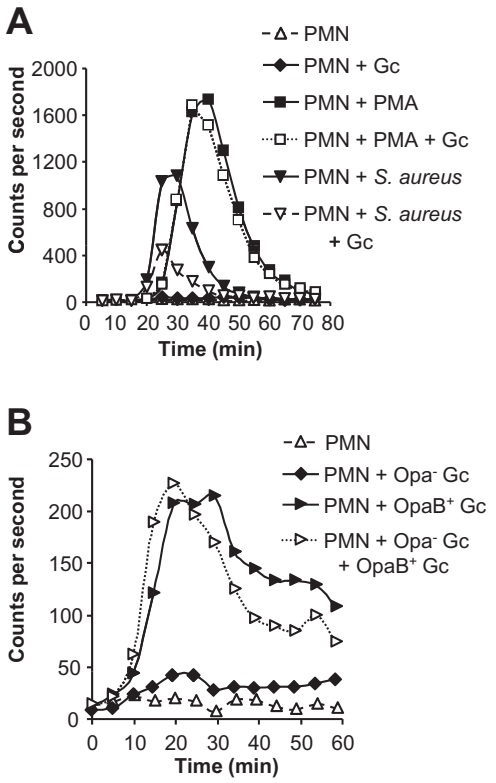

Fig. 6. Live Opa⁻ Gc do not suppress the oxidative burst of PMNs stimulated with phorbol esters or Opa⁺ bacteria.
A. Gc do not reduce the PMA-induced PMN oxidative burst. PMNs were left untreated, stimulated with 1 ng ml^{-1} PMA or infected with opsonized *S. aureus* at an moi of 130. Fifteen minutes prior to PMA addition, a subset of PMNs were infected with Opa⁻ MS11 Gc at moi 100 as indicated. ROS production was measured as in Fig. 1A.
B. Opa⁻ Gc do not inhibit the small oxidative burst elicited by OpaB⁺ Gc. PMNs were left uninfected or were infected with live Opa⁻ FA1090 Gc (moi 130), live OpaB⁺ FA1090 Gc (moi 50), or Opa⁻ and OpaB⁺ Gc together.

induced burst by 65% under these conditions (Fig. 6A). Opa⁻ Gc also had no effect on the small oxidative burst elicited by OpaB⁺ Gc, even though almost threefold more Opa⁻ bacteria were present (Fig. 6B). Therefore, live, Opa⁻ Gc have the ability to suppress the PMN oxidative burst that is elicited by a subset of stimuli, which can be either soluble (fMLP) or particulate (*S. aureus*).

Inhibition of the PMN oxidative burst is contact-dependent

To begin to characterize the mechanism by which viable Opa⁻ Gc suppress the PMN oxidative burst, we examined whether suppression was mediated by secreted Gc products or required direct bacterial contact with PMNs. To test the former possibility, Opa⁻ MS11 Gc were grown to mid-logarithmic phase in a rich defined medium optimized for Gc growth (Morse and Bartenstein, 1980), and the spent supernatant was added to PMNs. The spent supernatant did not itself induce ROS production in PMNs (Fig. 7A), suggesting that any immunomodulatory products of Gc are not present in culture supernatants at sufficient quantities to activate PMNs on their own. Exposure to the spent supernatant had no effect on the PMN oxidative burst induced by *S. aureus* (Fig. 7A), whereas live, intact Gc grown in the defined medium inhibited the maximal *S. aureus*-induced burst by nearly 50% (Fig. 7A). Therefore, Gc do not secrete factors that modulate the PMN oxidative burst during growth in rich liquid medium.

In order to examine whether the suppressive factor was secreted specifically in response to PMN infection, PMNs were exposed to Opa⁻ MS11 Gc, and the supernatant from the infection was presented to naïve PMNs. PMN exposure to this conditioned supernatant did not

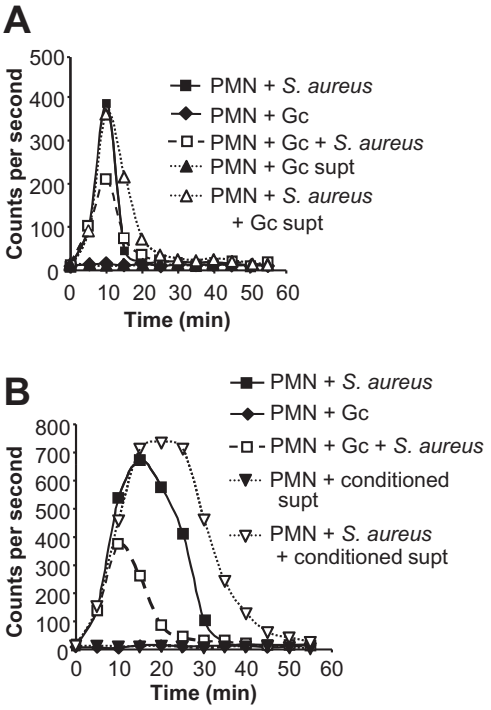

Fig. 7. Gc suppression of the PMN oxidative burst is not mediated by a secreted bacterial factor.
A. The suppressive factor is not directly secreted by live Opa⁻ Gc. PMNs were infected with Opa⁻ MS11 Gc (moi 35), opsonized *S. aureus* (moi 90), or *S. aureus* and Gc simultaneously. Other PMNs were exposed to the spent supernatant (supt) from liquid-grown, exponential-phase Gc, alone or in the presence of *S. aureus*.
B. The suppressive factor is not released from Gc or PMNs following infection. Conditioned supernatant (supt) was collected from PMNs infected for 1 h with MS11 Gc at an moi of 100 in the absence of luminol. Naïve PMNs were exposed to the conditioned supernatant in the absence or presence of opsonized *S. aureus* (moi 25). Other PMNs were infected with exponentially growing MS11 Gc (moi 140), *S. aureus*, or Gc and *S. aureus* simultaneously, without the addition of conditioned supernatant.
In (A) and (B), uninfected PMNs did not generate detectable ROS (data not shown).

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suppress the *S. aureus*-induced burst and in fact enhanced it, likely due to release of bacterial and/or PMN-derived stimulating factors into the infection medium (Fig. 7B). In the same experiment, intact Gc suppressed the *S. aureus*-induced burst by 63% (Fig. 7B). These results show that suppression of the PMN oxidative burst is not mediated by a secreted factor derived from either Gc or PMNs.

To test the alternative possibility that Gc-mediated inhibition of the PMN oxidative burst is contact-dependent, ROS production was monitored in PMNs separated from live Opa⁻ Gc by 0.2-μm-pore filter supports. When physically separated from PMNs in this manner, Gc did not inhibit ROS production in response to *S. aureus* (where *S. aureus* and PMNs were incubated on the same side of the filter support), and in fact enhanced the *S. aureus*-induced burst (Fig. 8A). The reason for this potentiation of the *S. aureus* burst is unclear but may be attributable to factors released by Gc growing in the Transwell system (but not sufficiently present in spent bacterial supernatants), which pass through the filter pores and enhance PMN ROS metabolism. In contrast, the same culture of liquid-grown, exponential-phase Gc inhibited the *S. aureus*-induced burst by 80% when the filter was absent (Fig. 8A). Therefore, Gc require contact with PMNs to inhibit the phagocyte oxidative burst.

As both adherent and phagocytosed Gc are found in association with PMNs during infection, we examined whether Gc needed to be internalized by PMNs to prevent ROS production by treating PMNs with the actin-depolymerizing toxin CD. CD shifted the kinetics of ROS production in response to fMLP such that the oxidative burst was initiated slightly earlier and persisted for less time than in untreated PMNs (Fig. 8B). The inhibition of ROS production in Gc-infected, fMLP-stimulated PMNs was similar whether CD was present or not, as infection reduced the fMLP-stimulated LDCL in untreated PMNs by 71% and in CD-treated PMNs by 61%. From this series of experiments, we conclude that live Gc suppress the PMN oxidative burst by production of a factor requiring *de novo* protein synthesis, whose mechanism of action requires bacterial contact with PMNs but is independent of phagocytosis of Gc.

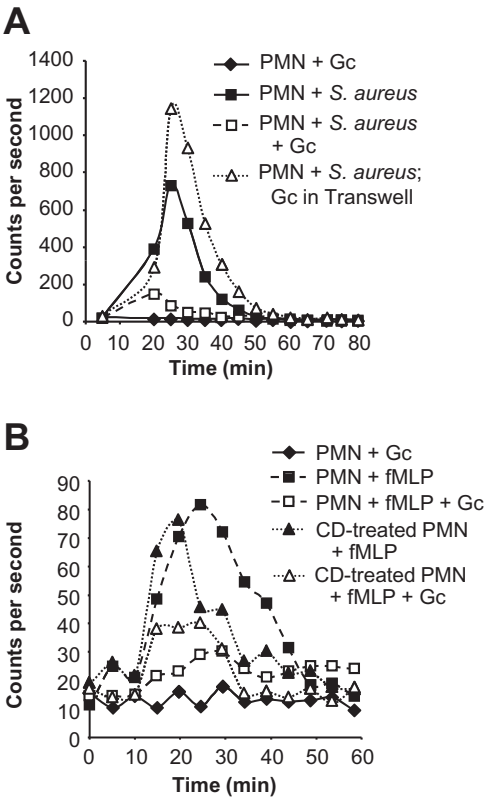

Fig. 8. Gc suppression of the PMN oxidative burst requires bacteria–PMN contact but not phagocytosis.
A. Gc inhibition of PMN ROS production requires bacteria–PMN contact. PMNs were incubated with Opa⁻ MS11 Gc (moi 200) for 15 min, where Gc and PMNs were allowed to mix in the well (PMN+ Gc) or were separated by a 0.2-μm-pore filter support (Gc in Transwell). Opsonized *S. aureus* (moi 40) were then added to the same side of the wells as PMNs. ROS production was measured as in Fig. 1A.
B. Extracellular Gc suppress the PMN oxidative burst. PMNs were treated with 10 μg ml^{-1} cytochalasin D (CD) or an equal volume of DMSO carrier. PMNs were then infected with liquid-grown Opa⁻ MS11 Gc (moi 320), treated with 10 μM fMLP, or exposed to Gc and fMLP simultaneously, in the absence or presence of CD as indicated.
In (A) and (B), uninfected PMNs did not generate detectable ROS (data not shown).

Discussion

The long-term association of Gc with the human population reflects its successful adaptation to life exclusively in the human urogenital tract. Although the controlled environmental and nutritional conditions of this anatomic site support bacterial growth and provide a direct route for the spread of infection, Gc must also contend with the potent PMN-rich innate immune response initiated in response to bacterial colonization. This work reveals one previously unappreciated means by which Gc subvert PMN antimicrobial activity: the ability of live, exponentially growing bacteria to actively suppress the PMN oxidative burst. Disarming this critical aspect of PMN defences may enhance Gc virulence in infectious exudates to facilitate colonization of the current host and transmission to new individuals.

Several lines of evidence indicate that Gc suppression of PMN ROS production is an active process. First, heat-killed or plate-grown bacteria retain the ability to stimulate ROS production in PMNs, even in the absence of serum opsonins. This finding implies that PMNs have the ability to recognize and respond to Gc products; however, live

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bacteria overcome their stimulatory effects. Stimulatory products of Gc could include lipooligosaccharide, peptidoglycan, porins and the Lip lipoprotein, which are recognized by Toll-like receptors that are expressed on many cell types including PMNs (Massari *et al*., 2002; Fisette *et al*., 2003; Mogensen *et al*., 2006). Second, bacterial protein synthesis is required for Gc to block the PMN oxidative burst, as demonstrated using sublethal concentrations of antibiotics that impede ribosomal function. Third, PMNs retain viability and the ability to respond to phorbol esters during infection with Gc. The latter observation also implies that live, exponentially growing Gc did not deplete the assay medium of dissolved oxygen, the substrate for NADPH oxidase activity, even if Gc oxygen consumption is stimulated by the presence of phagocyte-derived lactate (Britigan *et al*., 1988). Fourth, Gc do not immediately detoxify the ROS produced by PMNs by virtue of their potent catalase activity, as catalase-deficient Gc also fail to elicit the PMN oxidative burst. Taken together, these observations indicate that viable Gc synthesize factors that prevent PMNs from producing ROS in response to pathogen-associated molecular patterns (PAMPs). PAMPs are present not only on Gc but also on the Gram-positive *S. aureus* and are mimicked by the formylated peptide fMLP, two stimuli whose PMN oxidative burst was inhibited by Gc. Given these observations, we hypothesized that *S. aureus* survival after infection of PMNs would be enhanced if Gc were also present. Using a previously published PMN assay (Stohl *et al*., 2005), our results suggest that co-infection with Gc does increase *S. aureus* survival, but the effect is modest (twofold increase in recovered *S. aureus*) and variable among experiments, suggesting that only a subset of *S. aureus* are killed by oxidative means in PMNs (data not shown). It will be intriguing to determine whether Gc modulate the survival of pathogens that commonly cocolonize the urogenital tract when confronted with activated, ROS-producing PMNs.

N. gonorrhoeae joins a number of pathogens that are known to modulate PMN oxidative metabolism, but this bacterium appears to utilize a unique mechanism to accomplish this feat. Unlike *Helicobacter pylori*, *Salmonella enterica* serovar Typhimurium, *Streptococcus pyogenes* and *Leishmania donovani*, which stimulate the PMN oxidative burst but redirect NADPH oxidase activity away from the phagosome (Vazquez-Torres *et al*., 2000; Gallois *et al*., 2001; Allen *et al*., 2005; Lodge *et al*., 2006; Staali *et al*., 2006), viable Opa$^-$ Gc prevent PMNs from producing ROS. *Anaplasma phagocytophilum* and *Francisella tularensis* also block PMN oxidative metabolism, but these organisms suppress the respiratory burst in response to both fMLP and PMA, while Gc cannot block the PMA-induced burst (Mott and Rikihisa, 2000; Carlyon and Fikrig, 2003; Carlyon *et al*., 2004; McCaffrey and Allen, 2006). Failure to block the PMA-induced burst implies that Gc suppression of the oxidative burst does not involve proteolysis of NADPH oxidase subunits or transcriptional repression of oxidase genes, as associated with *A. phagocytophilum* infection (Carlyon *et al*., 2002; Mott *et al*., 2002). As PMA directly activates protein kinase C isoforms, these results also imply that suppression by Gc does not involve direct inhibition of protein kinase C activity, as found for *P. aeruginosa*-secreted phospholipase (Terada *et al*., 1999). Instead, we hypothesize that Opa$^-$ Gc infection blocks a signalling pathway that is required for NADPH oxidase activation, which is stimulated by engagement of the formyl peptide and complement receptors but not CEACAM receptors. These pathways could involve mitogen-activated protein kinases or protein kinase A, which are targeted by *Bacillus anthracis* toxins to suppress the PMN oxidative burst in response to fMLP but not PMA (Crawford *et al*., 2006), or tyrosine kinases or lipid second messengers that are required for fMLP-mediated NADPH oxidase activation (Le *et al*., 2002).

Although our results show that exponentially growing FA1090 OpaB$^+$ Gc elicit a minor respiratory burst in PMNs, others have reported that Opa-expressing Gc induce a potent phagocyte oxidative burst (Rest *et al*., 1982; Virji and Heckels, 1986; Fischer and Rest, 1988; Naids and Rest, 1991; Gray-Owen *et al*., 1997; Simons *et al*., 2005). In this study, Gc were grown to mid-logarithmic phase after successive rounds of subculturing into rich liquid medium, which allows $>95\%$ bacterial viability (data not shown). In previous reports where Gc stimulated the PMN oxidative burst, bacteria either were directly harvested from agar-containing medium or were harvested from agar medium and grown in liquid medium for short times. In both cases, dead bacteria and bacterial products are present along with live Gc. As we have shown that dead Gc (both Opa$^-$ and Opa$^+$) potently stimulate the release of ROS from PMNs, it is likely that previous studies were primarily measuring the effects of non-viable bacteria or shed bacterial products on PMNs. Intriguingly, these results raise the possibility that when PMNs are infected with live Gc in the presence of a stimulus known to induce an oxidative burst (e.g. dead bacteria or PMA), the resulting oxidative damage could cause increased bacterial killing. However, using a previously developed assay for measuring Gc survival after exposure to PMNs (Stohl *et al*., 2005), we have not found PMA-stimulated PMNs to kill liquid-grown Gc any more effectively than untreated PMNs (data not shown). These results may reflect the extensive and redundant antioxidant defences possessed by Gc (Seib *et al*., 2006). Alternatively, they may indicate that PMNs primarily direct non-oxidative antimicrobial mechanisms against Gc. This possibility is supported by reports from the Rest labora-

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tory showing that PMNs maintained under anoxic conditions or lacking NADPH oxidase activity retain some ability to kill Opa$^+$ Gc (Rest *et al*., 1982; Frangipane and Rest, 1992), as well as the recent observation that Gc mutated in genes encoding various antioxidants are not compromised for survival after PMN challenge (Seib *et al*., 2005). We are currently re-examining which antimicrobial mechanisms PMNs direct against Gc during infection.

This work provides two key observations about the unknown factor produced by liquid-grown, exponential-phase Gc that inhibits PMN oxidative metabolism. First, as suppression of the PMN oxidative burst requires *de novo* bacterial protein synthesis, suppression is mediated either by a protein that is rapidly turned over or strongly induced during PMN infection or by a non-protein factor that requires *de novo* protein synthesis for its production or release. Second, suppression of the PMN oxidative burst is not mediated by secreted bacterial products, in agreement with the observations that Gc do not produce exotoxins or encode large numbers of functional secreted proteins in their genome (Ulsen and Tommassen, 2006; GenBank accession AE004969). Certain strains of Gc possess a gonococcal genetic island (GGI) encoding a type IV secretion apparatus that secretes DNA (Dillard and Seifert, 2001; Hamilton *et al*., 2005), but the fact that strains MS11 (GGI+) and FA1090 (GGI–) can both suppress the PMN oxidative burst indicates that the type IV secretion system is not involved in modulating PMN activation. Instead, our results strongly suggest that Gc inhibition of the PMN oxidative burst requires bacteria–phagocyte contact. As the FA1090 genome does not encode secretion systems that deliver virulence-associated proteins into the host cell cytosol, we assume that the Gc suppressive factor is exported to the bacterial surface, where it interacts with the phagocyte plasma membrane. Thus far we have tested and discounted several surface-exposed Gc virulence factors for their potential to suppress ROS production in PMNs, including type IV pili (pilin and PilQ secretin), the MtrCDE efflux pump, IgA protease and a Gc phospholipase D homologue (Fig. 1B and data not shown). In previous work from our laboratory, mutants in *ngo1686* and *recN* were shown to be more sensitive to PMN killing (Stohl *et al*., 2005), but these mutants had no effect on PMN ROS production (data not shown). Although we do not currently know the identity of the Gc suppressive factor, one possibility is that ROS production in PMNs is modulated by Gc porin, which has been shown to translocate from the bacterial surface into eukaryotic plasma and internal membranes (Massari *et al*., 2003). Intriguingly, Lorenzen *et al*. have shown that purified Gc porin inhibits the oxidative burst of PMNs, but in other studies, porin had no effect on PMN oxidative metabolism (Bjerknes *et al*., 1995; Bauer *et al*., 1999; Lorenzen *et al*., 2000). We are exploring whether porin and other Gc products play a role in suppression of the PMN oxidative burst in this system.

Given that Gc are constantly barraged by the PMN-rich host inflammatory response, it is not surprising that this strict human pathogen has evolved mechanisms to withstand interactions with PMNs, including the ability to suppress the phagocyte oxidative burst. As the suppressive activity of Gc on PMN ROS metabolism varies depending on the viability of the bacteria and the surface structures present (i.e. Opa proteins), we speculate that Gc suppression of the PMN oxidative burst aids bacterial survival at specific stages of gonorrhoeal disease. Early in infection, when the majority of Gc may be viable, suppression of the oxidative burst would aid in colonization of the urogenital mucosa during the initial inflammatory response. The ability of Opa$^-$ Gc to suppress PMN oxidative metabolism may also allow the bacteria to ascend the urogenital tract and disseminate to secondary sites, as the majority of Gc isolates from the upper urogenital tract and those causing gonococcal arthritis lack Opa expression (O'Brien *et al*., 1983; Edwards and Apicella, 2004). In contrast, during active lower urogenital tract infection, the predominance of Opa$^+$ Gc (James and Swanson, 1978; Swanson *et al*., 1988; Jerse *et al*., 1994), in combination with increasing numbers of dead Gc and pro-inflammatory bacterial products, could overcome the suppression mediated by live Opa$^-$ Gc and stimulate the PMN oxidative burst. However, the intrinsically high resistance of Gc to ROS (Archibald and Duong, 1986; Hassett *et al*., 1990) as well as the ROS-mediated upregulation of gene products that protect Gc from PMN killing (Stohl *et al*., 2005) would aid Gc survival when confronted with the resulting PMN oxidative burst. Gc suppression of PMN oxidative metabolism may also help to explain the discrepancies in disease presentation between men and women. Male urethral infection selects for Opa$^+$ Gc that are capable of inducing ROS production in PMNs and may exacerbate the potent inflammatory response that characterizes acute disease in men (James and Swanson, 1978; Zak *et al*., 1984). In contrast, Opa$^-$ Gc predominate in women depending on the stage of the menstrual cycle, and suppression of the PMN oxidative burst under these conditions may contribute to the preponderance of asymptomatic or subclinical disease in the female population (James and Swanson, 1978; Edwards and Apicella, 2004). Given natural variations in Opa expression and the balance between viable and non-viable Gc during the course of human infection, Gc suppression of the PMN oxidative burst can be considered a dynamic process, serving at critical junctures to enhance gonorrhoeal pathogenesis in the face of the inflammatory host immune response.

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Experimental procedures

Bacterial strains and growth conditions

The parent strains of Gc used in this study were piliated, 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). Gc were maintained on gonococcal medium base agar (GCB) plus Kellogg's supplements (Kellogg *et al.*, 1963) and routinely grown for 20 h at 37°C, 5% CO_2. Constitutively non-piliated derivatives of FA1090 and MS11 carrying deletions of the *pilE* locus have been described (Segal *et al.*, 1985; Chen *et al.*, 2004). The deletions were introduced into the FA1090 1-81-S2 or MS11 VD300 chromosome by natural transformation, non-piliated progeny were identified by colonial morphology, and incorporation of the deletion was confirmed by Southern blotting with a *pilE*-specific probe. The FA1090 *kat::kan* mutant was obtained from A. Jerse (Uniformed Services University of the Health Sciences; Soler-Garcia and Jerse, 2004), the strain FA19 *mtrC::kan* mutant was obtained from W. Shafer (Emory University; Hagman *et al.*, 1995), the strain 1291 *pld::kan* mutant was obtained from M. Apicella and J. Edwards (University of Iowa and Research Institute at Nationwide Children's Hospital; Edwards *et al.*, 2003), and the FA1090 *pilQ::cat* mutation has been described (Long *et al.*, 2003). The mutations were introduced into FA1090 1-81-S2 Gc and/or MS11 VD300 Gc by natural transformation, and transformants were selected on the appropriate antibiotics at the following concentrations: kanamycin, 40 μg ml^{-1}; chloramphenicol, 0.6 μg ml^{-1} for FA1090 and 2 μg ml^{-1} for MS11. Genotypes were confirmed by Southern blot with gene-specific probes. In all strains, *pilE* genes were sequenced as described (Seifert *et al.*, 1994) to confirm retention of the desired pilin variant.

An OpaB-expressing (OpaB$^+$) derivative of FA1090 1-81-S2 Gc was isolated after *in vitro* passage. Isolates of FA1090 Gc in which all 11 *opa* genes were deleted and where *opaB* was re-introduced at a second site in the chromosome were provided by J. Cannon (University of North Carolina; Fulcher, 2004). Opa expression profiles of FA1090 and MS11 Gc were confirmed by immunoblotting bacterial lysates according to published methods (Black *et al.*, 1984) with a panel of monoclonal and polyclonal antibodies obtained from J. Cannon, M. Blake (FDA) and A. Jerse.

Viable, exponentially growing Gc were generated in liquid culture using a modification of a previously described technique (Stohl *et al.*, 2005). Gc cultured for 9–10 h on GCB were suspended at $OD_{550} = 0.07$ in gonococcal liquid medium containing Kellogg's supplements and 0.042% Na_2HCO_3 (GCBL) and grown at 30°C with rotation for 14–16 h. The cultures were diluted into fresh GCBL and grown at 37°C to $OD_{550} = 0.6–0.7$, then diluted to $OD_{550} = 0.07$ and grown at 37°C to mid-logarithmic phase. The cfu per ml present in each culture was estimated from a previously determined growth curve and validated by serial dilution and plate count. For experiments with plate-grown bacteria, confluent lawns of Gc that had been grown on GCB for 20 h were collected with a Dacron swab and re-suspended in liquid medium. To kill liquid-grown Gc, bacteria were heated at 56°C for 30 min or incubated in 70% isopropanol for 5 min. Gc protein synthesis was inhibited by treating liquid-grown FA1090 bacteria with 2 μg ml^{-1} chloramphenicol or 1 μg ml^{-1} tetracycline, concentrations that enrich for fully replicated chromosomes in Gc but do not affect bacterial viability (Tobiason and Seifert, 2006; and data not shown).

Staphylococcus aureus ATCC strain 25923 was obtained from K. Fullner-Satchell (Northwestern University) and grown on Todd–Hewitt agar (Difco). Overnight cultures of *S. aureus* in Todd–Hewitt broth (Difco) were diluted 1:100 into fresh broth and grown for 4–6 h. *S. aureus* were opsonized in 10% autologous human serum for 20 min at 37°C prior to PMN infection.

Pseudomonas aeruginosa strain PA99 expressing the type III secreted protein ExoU was obtained from A. Hauser (Northwestern University). Overnight cultures of PA99 in Luria–Bertani broth (LB; Difco) were diluted 1:40 into fresh LB and grown for 3 h.

Isolation of human PMNs

Heparinized venous blood was obtained from consented healthy volunteers following a protocol approved by the Northwestern University Institutional Review Board. Dextran-sedimented PMNs were purified on a Ficoll–Hypaque gradient as previously described (Stohl *et al.*, 2005). PMNs were re-suspended at 2×10^7 cells per ml in Dulbecco's PBS (without calcium and magnesium; Mediatech) containing 0.1% dextrose and kept on ice until use. PMN preparations routinely contained > 95% granulocytes, assessed morphologically by phase-contrast microscopy, and were >99% viable, monitored by trypan blue exclusion.

Luminol-dependent chemiluminescence (LDCL)

A total of 10^6 PMNs were incubated in Morse's defined medium (MDM; Morse and Bartenstein, 1980) in the presence of 20 μM luminol (Sigma), at a final volume of 1 ml. PMNs were infected with Gc at the indicated moi; the moi for heat-killed bacteria was determined by serial dilution and plate count of the culture prior to heat treatment. PMNs were stimulated with opsonized *S. aureus* at the indicated moi, 10 μM fMLP (Sigma) and/or 1–10 ng ml^{-1} PMA (Sigma), in the presence or absence of Gc. LDCL was measured in a LKB-Wallac 1250 luminometer (Perkin Elmer) every 5 min for 1 h at 37°C, and the counts per second (CPS) of LDCL were averaged per condition between duplicate wells. Untreated and 10 ng ml^{-1} PMA-treated PMNs served as negative and positive controls for ROS production respectively. In some experiments, PMNs were pre-exposed to 10 μM diphenylidene iodonium hydrochloride (Sigma) or 10 μg ml^{-1} CD (Sigma) for 10 min. Experiments were repeated at least three times, using PMNs from a different donor for each replicate.

For experiments with Transwells, PMNs were separated from Gc using 6.5-mm-diameter, 0.2-μm-pore Transwell filter supports (Costar). Opsonized *S. aureus* were added to the same side of the support as the PMNs to stimulate LDCL production. To collect spent Gc supernatants, liquid-grown MS11 VD300 Gc were prepared as described, except Gc were grown in MDM for the final subculture. Bacteria were pelleted from the mid-logarithmic-phase culture, and the supernatant was passed through a 0.22-μm-pore filter in order to remove residual bacteria. The supernatant was used in place of MDM in the LDCL assay. To collect culture supernatants from previously infected PMNs, 10^6 PMNs were infected with Gc at an moi of 100 for 1 h in MDM. The supernatant was collected and passed through a 0.2 μm syringe filter, added to naïve PMNs in the presence of luminol and *S. aureus*, and LDCL was measured for 1 h as above.

Total LDCL was calculated by approximating the area under the curve by the trapezoidal rule, i.e. the sum of LDCL produced in each interval between the times t_n and t_{n+1}, calculated as

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$$\sum (t_{n+1} - t_n) \times (\mathrm{CPS}_{tn+1} + \mathrm{CPS}_{tn}) \div 2$$

where CPS_{tn+1} and CPS_{tn} are the background-subtracted CPS of LDCL measured at each time point (background = unstimulated PMNs).

LDH assay

Polymorphonuclear leukocytes were re-suspended in modified Hepes-buffered saline (15 mM Hepes pH 7.4, 8 mM glucose, 4 mM KCl, 140 mM NaCl, 0.5 mM $MgCl_2$, 0.9 mM $CaCl_2$) and exposed to MS11 Gc at an moi of 390 for 1 h or left untreated. Release of LDH from PMNs was measured using the CytoTox 96 Non-Radioactive Cytotoxicity Assay kit (Promega) according to the manufacturer's instructions. PMNs infected for 3 h with ExoU-expressing *P. aeruginosa* at an moi of 100 served as a positive control for PMN lysis. Experiments were repeated three times, using PMNs from different donors, with similar results.

Immunofluorescence microscopy

Polymorphonuclear leukocytes were infected for 30 min with piliated, Opa$^-$ FA1090 Gc at an moi of 10. PMNs were fixed in 4% paraformaldehyde (Electron Microscopy Sciences) in PBS, and internal and external bacteria were discriminated from one another using a polyclonal anti-*N. gonorrhoeae* antibody (Biosource) and a differential immunofluorescence procedure as previously described (Criss and Seifert, 2006). Cells were examined using a LSM510 confocal laser scanning microscope (Zeiss). Images were acquired with LSM510 operating software and processed with Adobe Photoshop 7.0.

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