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Investigation of Oxidative Stress Defenses of *Neisseria gonorrhoeae* by Using a Human Polymorphonuclear Leukocyte Survival Assay

Kate L. Seib,[1] Mark P. Simons,[2] Hsing-Ju Wu,[3] Alastair G. McEwan,[1] William M. Nauseef,[2] Michael A. Apicella,[2] and Michael P. Jennings[1]*

The School of Molecular and Microbial Sciences and The Centre for Metals in Biology, The University of Queensland, Brisbane, Australia[1]; Department of Microbiology, Inflammation Program, and Department of Medicine, University of Iowa, Iowa City, Iowa[2]; and Core Facilities for Proteomics Research, Institute of Biological Chemistry, Academia Sinica, Taipei, Taiwan[3]

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Neisseria gonorrhoeae **has well-characterized oxidative stress defense systems that protect against oxidative killing in in vitro assays. In contrast, mutant strains of *N. gonorrhoeae* lacking oxidative stress defenses are identical to the wild type when tested in an ex vivo survival assay using human polymorphonuclear leukocytes.**

Neisseria gonorrhoeae, the causative agent of the sexually transmitted infection gonorrhea, is a host-adapted pathogen that poses serious health implications. Gonococcal infection is typically characterized by a symptomatic inflammatory response of the urogenital tract, which involves accumulation of activated polymorphonuclear leukocytes (PMNs) (4). The PMN-mediated inflammatory response involves migration of PMNs towards sites of infection, phagocytosis of microorganisms, and elimination of these organisms by oxygen-dependent and oxygen-independent mechanisms (reviewed in references 11 and 23).

Stimulation of PMNs results in a rapid increase in oxygen consumption referred to as the "oxidative burst." Assembly and activation of the NADPH oxidase in the plasma membrane results in the generation of superoxide (5), which rapidly dismutates to hydrogen peroxide (27) and is then consumed by myeloperoxidase, generating hypochlorous acid (52). Nitric oxide is also produced by constitutive and inducible nitric oxide synthases of PMNs (12, 25, 28). Various secondary oxidants are generated from these reactive species, including chloramines, hydroxyl radicals, singlet oxygen, and peroxynitrite (12, 23). Concurrent with the oxidative burst, intracellular granules fuse with the plasma membrane or phagosomal membrane to release a broad array of biologically active molecules, including proteases and antimicrobial proteins (reviewed in reference 18).

Without antibiotic treatment, gonococcal infections are persistent and resolve slowly (33), indicating that the PMN response is relatively ineffective in eradicating infection. However, interaction of *N. gonorrhoeae* with PMNs has been a controversial topic; some studies have reported that *N. gonorrhoeae* cells survive and grow within PMNs, while other studies have reported that *N. gonorrhoeae* is rapidly killed within PMNs (reviewed in references 37 and 44). The majority of these studies were performed using a tumbling tube assay with nonadherent PMNs. The use of adherent PMNs mimics the natural disease process in which PMNs have migrated from the bloodstream and are attached to cells and extracellular matrix proteins present at the site of infection. PMNs adhered to plates coated with serum or extracellular matrix proteins produce a large respiratory burst in response to stimuli, whereas suspension PMNs are relatively unresponsive (32). A recent study investigating interactions between *N. gonorrhoeae* and adherent human PMNs found the PMNs generated a substantial respiratory burst in response to gonococci (45). Despite this, a significant proportion of phagocytosed *N. gonorrhoeae* cells survived PMN killing and replicated over time (45). Viable counts and microscopic analysis indicated that some level of killing occurs after ingestion, but a subpopulation of *N. gonorrhoeae* cells survive and replicate (75.0% ± 18.31% at 1 h and 80.21% ± 15.34% at 2 h), in contrast to efficient killing of *Escherichia coli* (1.83% ± 0.36% at 1 h and 1.37% ± 0.08% at 2 h) (45).

It seemed probable that the ability of *N. gonorrhoeae* cells to survive in the hostile environment of the PMN would be due to the diverse array of oxidative stress defenses that this organism possesses. These defenses have typically been characterized based on the sensitivity of mutant strains to in vitro oxidative killing (see Table 1 for a summary). *N. gonorrhoeae* possesses one superoxide dismutase (Sod), which is an inactive or weakly active cytoplasmic SodB (4, 24, 53) that does not protect against reactive oxygen species in vitro (49). However, accumulation of manganese by the MntABC transport system confers protection against oxidative stress (49). *N. gonorrhoeae* also has high catalase and peroxidase activities (4, 24, 53), afforded by the cytoplasmic catalase (KatA) (4) and the periplasmic cytochrome *c* peroxidase (Ccp), both of which protect against hydrogen peroxide in vitro (42, 50). Other oxidative defenses described for *N. gonorrhoeae* include the following: a potential thiol-disulfide oxidoreductase, Sco (41); methionine sulfoxide reductase (MsrA/B) (46); and the iron storage protein bacterioferritin (Bfr) (14). Regulators of these defenses include the peroxide-responsive repressors of KatA and MntC, OxyR (48) and PerR (H.-J. Wu, K. L. Seib, Y. N. Srikhanta, J. L. Edwards, S. P. Kidd, M. A. Apicella, A. G. McEwan, and M. P. Jennings, submitted for publication), respectively.

* Corresponding author. Mailing address: The School of Molecular and Microbial Sciences, The University of Queensland, Brisbane 4072, Australia. Phone: 61 7 3365 4879. Fax: 61 7 3365 4620. E-mail: jennings@uq.edu.au.

TABLE 1. Strains used in the PMN phagocytosis assay and a summary of results

Strain	Description of strain	In vitro sensitivity[a]		PMN sensitivity[c]
		Sensitivity (substance)	Reference	
N. gonorrhoeae 1291	Wild type			−
sodB	Sod deficient	X (PQ, X/XO)	49	−
mntC	Lacks periplasmic Mn-binding protein of MntABC transporter (decreased [Mn] in cells)	S (PQ)	49	−
katA	Catalase deficient	S (H_2O_2)	42	−
ccp	Cytochrome *c* peroxidase deficient	S (H_2O_2 [slight])	42	−
ccp katA	Ccp and catalase double mutant	S (H_2O_2)	42	−
sco	Sco deficient (potential thiol-disulfide oxidoreductase)	S (PQ)	41	−
sco katA	Sco and catalase double mutant	S (PQ, H_2O_2)	41	−
oxyR	Overexpresses KatA	R (X/XO, H_2O_2)	48	−
perR	Overexpresses MntC (increased [Mn] in cells)	R (H_2O_2)	—[b]	−
E. coli DH5α	Wild type			+

[a] The strains used in the PMN phagocytosis assay are shown along with a summary of the results from this assay and results from in vitro assays from previous publications. Sensitivities of mutant strains to in vitro oxidative stress killing assays are shown relative to *N. gonorrhoeae* strain 1291 (wild type): X, same phenotype as wild type; S, sensitive to killing; R, resistant to killing (increased survival relative to wild type). These assays involved exposure of a suspension of 10^4 to 10^6 cells to either paraquat (PQ; 10 mM), xanthine (4.3 mM)/xanthine oxidase (300 mU/ml) (X/XO), or hydrogen peroxide (H_2O_2; 10 or 40 mM). For further details, see the references cited in the table.

[b] —, H.-J. Wu, K. L Seib, Y. N. Srikhanta, J. L. Edwards, S. P. Kidd, M. A. Apicella, A. G. McEwan, and M. P. Jennings, submitted for publication.

[c] PMN sensitivity (+) is defined as a statistically significant difference in the mean percent survival of the strain relative to *N. gonorrhoeae* strain 1291 (wild type) in the PMN phagocytosis assay. *P* values that are <0.05 are considered statistically significant. Differences between *N. gonorrhoeae* 1291 and its mutant derivatives were not statistically significant (−) (Fig. 1) (P values ≥ 0.14).

The oxidative stress that *N. gonorrhoeae* encounters within the host environment is significantly more complex than that generated by in vitro assays. Indeed, the expression of numerous genes involved in defense against oxidative stress is upregulated in *E. coli* upon exposure to PMNs (47). Many of these defenses (i.e., catalase, Sod, and OxyR) have been shown to protect enteric bacteria against killing by activated PMNs (19, 29, 39, 47). The role of oxidative defenses in protecting *N. gonorrhoeae* from the PMN oxidative burst was determined using the phagocytosis assay described by Simons et al. (45). Briefly, approximately 2×10^6 PMNs isolated from peripheral blood were adhered to glass coverslips pretreated with collagen and autologous human serum. *N. gonorrhoeae* strain 1291 (wild type) and a set of mutant derivatives (Table 1) expressing opacity-associated protein (Opa^+) and pili (P^+) (as determined by colony morphology) were grown to log phase. Bacteria were added to PMNs at a multiplicity of infection of 1:1 and centrifuged, and the supernatant was removed (time A). Synchronized phagocytosis was stimulated by the addition of warm medium for 2 minutes (time B). Nonphagocytosed bacteria were removed by three wash steps (time 0 h). PMNs and bacteria were incubated at 37°C for 1 hour (time 1 h). Saponin was used to lyse PMNs, and viable CFU were enumerated after overnight culture of plated serial dilutions. Each assay was done with triplicate cultures of each mutant strain along with wild-type controls and was performed on at least three occasions. Laser scanning confocal microscopy, scanning electron microscopy, and transmission electron microscopy performed by Simons et al. (45) illustrated that PMN-associated *N. gonorrhoeae* cells were internalized and contained within defined phagosomes within the perimeter of the PMN membrane and that there were very few bacteria residing in the extracellular spaces.

This study is based on a set of previously constructed and defined mutant strains of *N. gonorrhoeae* that are deficient in various oxidative stress defense mechanisms or regulatory systems (see Table 1 and references cited therein for further detail), with the exception of the *sco katA* mutant strain, which was constructed by transforming the *sco*::*kan* plasmid (41) into the *katA*::*tet* strain (42). All mutant strains were confirmed by Southern hybridization, PCR, and/or sequence analysis. The set of mutant strains was compared to wild-type strain 1291 to determine relative sensitivity to killing by PMNs (Fig. 1). A summary of results is shown in Table 1, along with results of in vitro assays from previously published work. Surprisingly, none of the mutant strains tested had a phenotype distinct from the wild type in the PMN phagocytosis assay. Wild-type and mutant strains had similar levels of PMN association (time A) and phagocytosis (time B), indicating the mutations had no detectable effect on phagocytosis by PMNs.

N. gonorrhoeae is a host-adapted pathogen which is able to survive killing by PMNs. Based on the broad range of oxidative stress defenses that *N. gonorrhoeae* cells possess, it was proposed that the ability to survive within PMNs was dependent, at least in part, on protection against the oxidative burst. This hypothesis is not supported by the similar phenotypes that were observed for the wild-type strain and the oxidative stress defense mutant strains in the PMN assay.

Several species of bacteria are able to survive PMN killing by inhibiting or subverting the oxidative burst of PMNs (reviewed in reference 1). As seen with *N. gonorrhoeae* in this study, *Helicobacter pylori* is able to evade PMN killing despite the activation of an oxidative response. *H. pylori* alters the targeting of the PMN NADPH oxidase so that it locates to the plasma membrane rather than the phagosomal membrane, thus releasing reactive oxygen species into the extracellular environment (2).

The features that govern *N. gonorrhoeae*-PMN interactions have not yet been defined, and while interactions do involve the gonococcal surface proteins porin (8, 21, 22, 26, 31) and Opa (7, 20, 31, 51), their roles in stimulating versus inhibiting the oxidative burst of PMNs have not been resolved. Further investigation using the adherent PMN phagocytosis assay (45) should provide a better understanding of the *N. gonorrhoeae*-

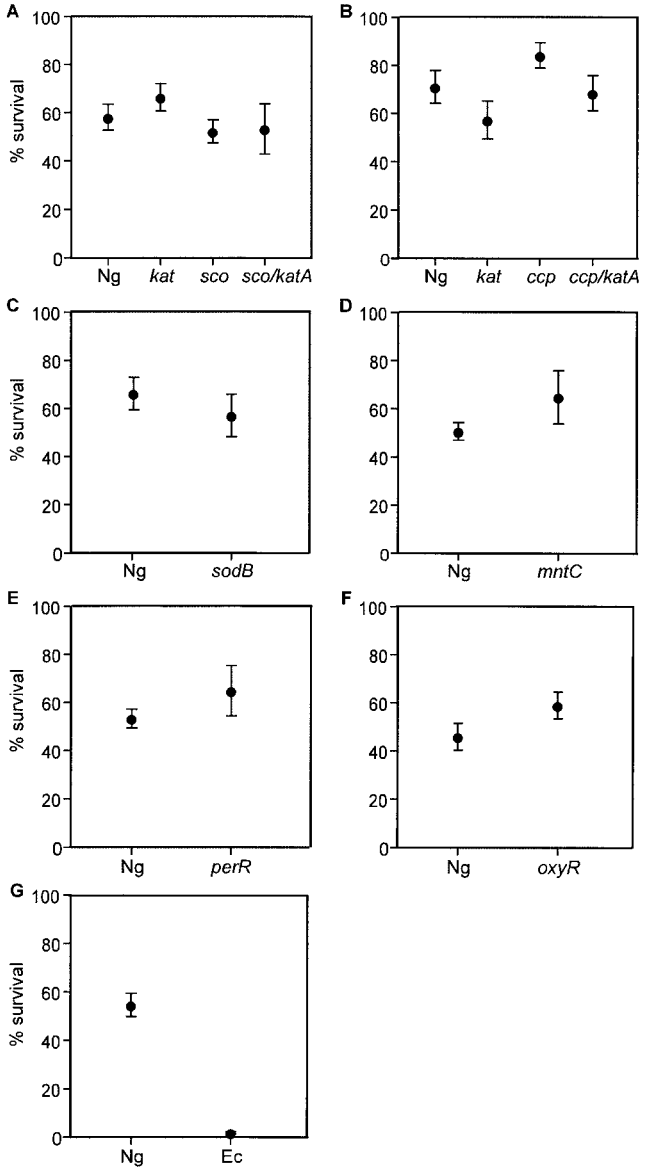

FIG. 1. PMN phagocytosis assay. PMNs were incubated with *N. gonorrhoeae* wild-type strain 1291 and mutant derivatives deficient in various oxidative stress defense mechanisms or regulatory systems to determine relative sensitivity to killing by PMNs (as described by Simons et al. [45]). Briefly, bacteria were added to a monolayer of PMNs (approximately 2×10^6) at a multiplicity of infection of 1:1 and incubated at 37°C for 1 h. PMN were then lysed to quantify CFU. Each panel (A to G) shows a set of experiments in which wild-type strain 1291 (Ng) was compared to a mutant strain, a set of mutant strains, or *Escherichia coli* strain DH5α (Ec), as shown on the *x* axis. Results shown are the percent survival of bacteria at time 1 h (expressed as a percentage of viable bacteria at time 0 h). Each data point is the mean of several individual experiments ± one standard deviation of the mean (number of repeat experiments: panel A, 3; B, 4; C, 2; D, 4; E, 2; F, 3; and G, 3) in which each experiment was conducted with three replicates of each strain. To determine whether the percent survival of mutant strains was statistically significant compared to the percent survival of the wild-type strain, *P* values were computed using unpaired two-sided Student's *t* test. *P* values that were less than 0.05 were considered statistically significant. Differences between *N. gonorrhoeae* wild-type strain 1291 and its mutant derivatives were not statistically significant (panels A to F, $P \geq 0.14$). The only statistically significant difference was between wild-type *N. gonorrhoeae* and *E. coli* (panel G; $P < 0.0001$).

dependent oxidative burst of PMNs. Despite the stimulation of an oxidative burst by *N. gonorrhoeae* (7, 31, 45, 51), the results described herein support previous studies which suggested that oxygen-independent mechanisms may be of greater significance than oxygen-dependent mechanisms during PMN killing of *N. gonorrhoeae* (9, 10, 13, 35, 36, 43; reviewed in reference 44).

The resistance of the specific mutant strains may also be due to the presence of redundant defenses. For example, in *E. coli*, a *sodA* or a *sodB* mutant strain is no more sensitive to PMN killing than the wild-type strain, but a s*odA sodB* double mutant is more susceptible to killing (29, 34). It is also important to note that the defenses investigated do not protect against all of the types of oxidants generated during the PMN oxidative burst (e.g., nitric oxide), some of which may be of greater importance in the killing of *N. gonorrhoeae*.

In light of our results, the presence of a wide range of defenses in *N. gonorrhoeae* suggests that this organism encounters significant oxidative stress from other sources in vivo. The primary sites of gonococcal infection are the ecto- and endocervical epithelium in women (17) and the urethral epithelium in men (3, 15). *N. gonorrhoeae* cells are able to survive and replicate within epithelial cells at these sites of infection (reviewed in reference 30). Intestinal and airway epithelial cells are able to kill bacteria by oxidative mechanisms (6, 16, 38, 40), and cervical epithelial cells also appear to have such oxidative defense capacity. We have recently observed that both MntC and PerR of *N. gonorrhoeae* are required for survival within primary human cervical epithelial cells (H.-J. Wu, K. L. Seib, Y. N. Srikhanta, J. L. Edwards, S. P. Kidd, M. A. Apicella, A. G. McEwan, and M. P. Jennings, submitted for publication).

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