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

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Neisseria gonorrhoeae has well-characterized oxidative stress defense systems that protect against oxidative killing 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 in 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 oxido reduction, 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.
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 up-regulated 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 pilus (P⁺) (as determined by colony morphology) were grown to log phase. Synchronized phagocytosis was stimulated by the addition of *N. gonorrhoeae* cells to either paraquat (PQ; 10 mM), xanthine (4.3 mM)/xanthine oxidase (300 mU/ml) (X/XO), or hydrogen peroxide (H₂O₂; 10 or 40 mM). For further details, see the references cited in the table.

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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description of strain</th>
<th>In vitro sensitivity</th>
<th>PMN sensitivity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>N. gonorrhoeae</em> 1291</td>
<td>Wild type</td>
<td>X (PQ, X/XO)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>sodR</td>
<td>Sod deficient</td>
<td>S (PQ)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>mntC</td>
<td>Lacks periplasmic Mn-binding protein of MntABC transporter (decreased [Mn] in cells)</td>
<td>S (PQ)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>katA</td>
<td>Catalase deficient</td>
<td>S (H₂O₂)</td>
<td>42</td>
<td>—</td>
</tr>
<tr>
<td>ccp katA</td>
<td>Cytochrome c peroxidase deficient</td>
<td>S (H₂O₂)</td>
<td>42</td>
<td>—</td>
</tr>
<tr>
<td>sco katA</td>
<td>Sco deficient (potential thiol-disulfide oxidoreductase)</td>
<td>S (PQ)</td>
<td>41</td>
<td>—</td>
</tr>
<tr>
<td>oxyR</td>
<td>Overexpresses KatA</td>
<td>S (PQ, H₂O₂)</td>
<td>41</td>
<td>—</td>
</tr>
<tr>
<td>perR</td>
<td>Overexpresses MntC (increased [Mn] in cells)</td>
<td>R (X/XO, H₂O₂)</td>
<td>48</td>
<td>—</td>
</tr>
<tr>
<td>E. coli DH5α</td>
<td>Wild type</td>
<td>R (H₂O₂)</td>
<td>—b</td>
<td>—</td>
</tr>
</tbody>
</table>

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 10⁶ to 10⁷ cells to either paraquat (PQ; 10 mM), xanthine (4.3 mM)/xanthine oxidase (300 mU/ml) (X/XO), or hydrogen peroxide (H₂O₂; 10 or 40 mM). For further details, see the references cited in the table.


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).

Several species of bacteria are 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 localizes to the plasma membrane rather than the phagosomal membrane, thus releasing reactive oxygen species into the extracellular environment.

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* interactions.
significant (panels A to F, wild-type strain 1291 and its mutant derivatives were not statistically considered statistically significant. Differences between difference was between wild-type N. gonorrhoeae survival of the wild-type strain, mutant strains was statistically significant compared to the percent replicates of each strain. To determine whether the percent survival of 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 edges The University of Queensland Graduate School for providing a 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).

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 X 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 DH5 /Ec/ (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 ≥ 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 sodA 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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