Neisseria gonorrhoeae Catalase Is Not Required for Experimental Genital Tract Infection despite the Induction of a Localized Neutrophil Response[▽]

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Received 20 September 2006/Returned for modification 6 December 2006/Accepted 24 January 2007

Neisseria gonorrhoeae produces several antioxidant defenses, including high levels of catalase, which may facilitate the persistence during an inflammatory response via neutralization of H₂O₂ produced by phagocytes. In vivo testing of the role of catalase in gonococcal survival is critical since several physiological factors impact interactions between *N. gonorrhoeae* and polymorphonuclear leukocytes (PMNs). Here we assessed the importance of gonococcal catalase in a surrogate model of female genital tract infection. Female BALB/c mice were treated with 17- β estradiol to promote susceptibility to *N. gonorrhoeae* and inoculated intravaginally with wild-type gonococci or a catalase (*kat*) deletion mutant. A localized PMN influx occurred in an average of 43 and 81% of mice infected with wild-type or *kat* mutant gonococci, respectively, and PMNs associated with numerous wild-type or catalase-deficient bacteria were observed in vaginal smears. The combined results of six experiments showed a significant difference in the number of days wild-type bacteria were recovered compared to the catalase-deficient gonococci. However, there was much variability between experiments, and we found no correlation between PMN influx, colonization load, and clearance of wild-type or *kat* mutant bacteria. Estradiol treatment did not impair bacterial uptake, the luminol-dependent chemiluminescence response, or the killing capacity of isolated murine PMNs against *N. gonorrhoeae* or *Staphylococcus aureus*. Our data suggest *N. gonorrhoeae* is not significantly challenged by H₂O₂ produced by PMNs in the murine lower genital tract; alternatively, redundant defense mechanisms may protect the gonococcus from reactive oxygen species during infection.

Neisseria gonorrhoeae is a common cause of uncomplicated mucosal infections of the human lower urogenital tract, pharynx, and rectum. More serious infections occur when the organism ascends to the upper reproductive tract, which in females can lead to pelvic inflammatory disease and the postinfection complications of infertility, ectopic pregnancy, and chronic pelvic pain. Many infections are silent; however, acute gonorrhea is characterized by an intense inflammatory exudate that contains macrophages, exfoliated epithelial cells, and numerous polymorphonuclear leukocytes (PMNs) (21). PMNs with intracellular diplococci are frequently observed in patient exudates, and although most gonococci appear to succumb to PMN killing after internalization, there is substantial evidence that a proportion of intracellular gonococci survives and even multiplies within the PMN (9, 10, 33, 41, 49, 50).

Professional phagocytes utilize both O₂-independent and O₂-dependent mechanisms to kill bacteria. An important arm of the O₂-dependent defense is the production of reactive oxygen species (ROS) via a cascade of reactions known as the phagocytic respiratory burst. Fueled by molecular O₂ and glu-

cose, the first step of the respiratory burst is the generation of superoxide anion (O₂⁻), which is catalyzed by NADPH oxidase within the phagocytic vacuolar membrane. O₂⁻ is rapidly converted to H₂O₂ in the presence of iron or spontaneously dismutates to H₂O₂ in the low pH of the phagocytic vacuole. H₂O₂ reacts with Fe²⁺ to produce OH[•] or with myeloperoxidase to generate hypochlorous acid (HOCl). The ROS produced by this chain of reactions are deleterious to microbes through their effects on DNA, protein, and other macromolecular structures (45).

N. gonorrhoeae is equipped with a battery of antioxidant factors that may neutralize ROS produced by phagocytes during infection (40). The increased sensitivity of genetically defined *kat* mutants to H₂O₂ or chemical inducers of oxidative stress (25, 43, 48) and the fact that this enzyme is produced at relatively high levels among bacteria (1, 20) suggests that catalase protects *N. gonorrhoeae* against the phagocytic respiratory burst. This hypothesis is indirectly supported by the detection of increased catalase activity in *N. gonorrhoeae* exposed to PMNs (54). Recently, however, Seib et al. (39) questioned the role of catalase in defense against PMN killing by demonstrating that *N. gonorrhoeae* mutants in *kat* and other antioxidant genes were not more susceptible to killing by human PMNs. Although this result may be predictive of the importance of gonococcal catalase in vivo, the use of in vitro assays to study the bacterial evasion of PMNs may be compromised by the difficulty in reproducing the intricate balance of bacterial and host factors in inflammatory foci. Most investigations of gono-

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[▽] Published ahead of print on 12 February 2007.

coccal evasion of PMN killing have been performed under conditions that do not reproduce the O_2 tension and iron concentration of specific body sites, which along with glucose are critical in dictating the potency of the respiratory burst. The design of appropriate assay conditions is further complicated by the dramatic effect of pH on O_2 consumption by PMNs (16) and the fact that lactate also influences the oxidative capacity of PMNs when in the presence of *N. gonorrhoeae* (8). In addition, sialylation of lipooligosaccharide (LOS), which in *N. gonorrhoeae* requires exogenous substrate provided artificially or by the host, should also be considered in the design of such studies since LOS sialylation clearly influences gonococcal interactions with phagocytes (18, 37, 52). Environmental stimuli may also influence the expression of catalase and other antioxidant factors (40), which may or may not be present in certain assay media.

Although several host restrictions limit the usefulness of nonhuman primates in studying gonococcal pathogenesis, *N. gonorrhoeae* can infect the lower genital tract of female mice provided they are given 17 β -estradiol to promote a proestrus, followed by an estrus-like state, which we (13) and others (6, 24) have shown to be the stages of the murine estrous cycle that are transiently susceptible to gonococcal colonization. The appropriateness of the mouse model for testing predictions about the role of gonococcal catalase in vivo includes the presence of several important similarities between the lower genital tracts of mice and women such as O_2 and iron concentrations, the presence of glucose and lactate (14), commensal flora (22, 23), and a vaginal pH in mice that is similar to that of the human endocervix (6). Importantly, ca. 50% of infected mice elicit a localized PMN response to gonococcal infection (22), gonococci become sialylated during murine infection, and interactions between mouse PMNs and sialylated gonococci closely parallel that which has been reported for human PMNs (52). Accordingly, here we focused on catalase as a potentially critical player in gonococcal survival during experimental genital tract infection of mice. We also examined the interactions between murine PMNs and *N. gonorrhoeae* to further define the usefulness of the mouse model for studying gonococcal evasion of an inflammatory response.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *N. gonorrhoeae* strain FA1090 (PorB.1B; Sm^r) was originally isolated from a female with disseminated gonococcal infection and has been shown to cause experimental urethritis in male volunteers (12). Strain GP501 (*kat* _{Δ 1.2 kb}) is a mutant of FA1090 in which the *kat* gene was inactivated via an in-frame deletion (43). The frozen stocks of wild-type and *kat* mutant gonococci used here consisted primarily of piliated, OpaB variants that expressed a single LOS species on an 8 to 16% gradient of sodium dodecyl sulfate-polyacrylamide gel as assessed by silver staining of proteinase K-treated bacterial lysates (46). All *Neisseria* strains were cultured in 7% CO_2 at 37°C on GC agar supplemented with Kellogg's supplement I and FeNO₃ as described previously (22). Supplemented GC broth with 5 mM NaHCO₃ was used for in vitro growth curves, which were determined with shaking under ambient conditions. GC-VCNTS (vancomycin, colistin, nystatin, trimethoprim sulfate, and streptomycin) agar was used to isolate *N. gonorrhoeae* from vaginal mucus. All media were from Difco Laboratories (Detroit, MI).

Isolation of murine PMNs. Murine PMNs were elicited by intraperitoneal injection of 2.5 ml of 3% thioglycolate broth (3) and collected by peritoneal lavage 5 h later using cold Hanks balanced salt solution (HBSS) and an 18-gauge needle. Thioglycolate broth was prepared in endotoxin-free water (29.5 g/liter); the broth was gently boiled for 20 min, autoclaved for 30 to 45 min, and stored at 4°C in 50-ml conical tubes after cooling. All glassware was baked overnight at

225°C and autoclaved for 1 h before use. For each experiment, PMNs from two to three mice were pooled, centrifuged, and washed as described previously (52) before resuspending them in 4 to 5 ml of HBSS complete buffer (HBSS with 10 mM glucose, 0.1% gelatin, 1 mM CaCl₂, and 1 mM MgCl₂). The final concentration of PMNs was determined by using a hemacytometer; trypan blue was used to confirm that >95% of the PMNs were viable.

Acridine orange-trypan blue staining. Gonococcal association with and internalization by PMNs were measured by the acridine orange fluorescence staining assay described by others (4) with the modification that trypan blue was used to visualize intracellular bacteria. Trypan blue is excluded by PMNs and thus quenches only the fluorescence of extracellular bacteria stained with acridine orange. Incubation of gonococci with PMNs was as described previously (52) except that phorbol myristate acetate (10 ng) was added 10 min before bacteria was added to the wells. Gonococci were preincubated with 10% normal mouse serum (NMS) or heat-inactivated mouse serum (HI-NMS) (56°C, 10 min) before it was added to the PMNs at a multiplicity of infection of 10:1 (bacteria to PMN). After 30, 60, 90, and 135 min of incubation at 37°C under 5% CO_2 , two sets of triplicate coverslips were washed with 1 ml of HBSS. One set was stained for 45 s with acridine orange (Difco), rinsed with HBSS, and mounted onto a slide. The second set was stained as described above, counterstained with 0.5 ml of 0.4% trypan blue for 45 s, and washed with HBSS. Inverted, stained coverslips were sealed onto glass slides with nail polish. One hundred PMNs were examined per coverslip by using a fluorescence microscope, and the number of PMNs with 0, 1 to 2, 3 to 10, or more than 10 bacteria was recorded. The average number of PMNs in each category was calculated from three coverslips stained with acridine orange alone or acridine orange plus trypan blue.

Opsonophagocytic killing assay. A modification of the "tumbling tube" protocol described by Rest et al. (36, 37) was utilized to assess PMN killing. Briefly, 1-ml suspensions containing 5×10^5 PMNs in complete HBSS buffer containing NMS or HI-NMS at a final concentration of 10% were pipetted into 2-ml polypropylene microcentrifuge tubes. Cell suspensions were placed on a rotary shaker in room air for 10 min at 37°C. Wild-type or GP501 bacteria (2.5×10^5 to 5.0×10^5 CFU) that were preincubated in 10% NMS or HI-NMS for 15 min were added to the PMNs. After 45, 90, and 135 min of incubation, 10- μ l samples were serially diluted in GC broth with 0.05% saponin and cultured on GC agar. Killing was measured by comparing the average number of CFU recovered from tubes containing NMS-opsonized bacteria to that from tubes containing bacteria incubated in HI-NMS. A control tube with no PMNs was included in each assay to assess bacterial viability over time. Serum was from mice treated with 17 β -estradiol; we have since found no difference in the degree of killing when serum from untreated mice is used. *Staphylococcus aureus* (strain RN6390B) was used to standardize the assay (provided by Guangyong Ji, Catholic University).

Chemiluminescence assay. The oxidative response of murine PMNs to *N. gonorrhoeae* was measured by luminol-dependent chemiluminescence (LDCL) (7, 29). Briefly, gonococci were opsonized for 15 min at 37°C in 10% NMS, centrifuged, and washed once with HBSS complete buffer. Murine PMNs were suspended at a concentration of 5×10^5 PMNs/ml in complete HBSS buffer and preincubated with luminol (0.1 mg/ml in HBSS) in a 5-ml scintillation vial for 2 min at 37°C (final volume, 1 ml). Bacteria were added at ratios ranging from 5:1 to 50:1 (bacteria to PMN). In some experiments, cytochalasin B (5 μ g/ml) was added to the PMNs. The production of primarily extracellular versus intracellular ROS was assessed by incorporating bovine catalase (2,000 U) or horseradish peroxidase (HRP; 4 U) and 1 mM NaN₃ to the reaction vials, respectively. Chemiluminescence signal was measured in a scintillation counter (model LS6100) at 0.2-min intervals using the preset full tritium window with the counter in normal in-coincidence mode for a defined period of time.

Experimental murine infection. Treatment of female BALB/c mice (6 to 8 weeks old; National Cancer Institute) with 17 β -estradiol and antibiotics was as described previously (22) except that a lower dose of streptomycin sulfate (0.24 mg, twice daily) was used. Mice were inoculated intravaginally with wild-type FA1090 or *kat* mutant GP501 bacteria (five to eight mice per group) harvested from GC agar into phosphate-buffered saline with 1 mM MgCl₂ and 2 mM CaCl₂ after 19 h of incubation. Inocula were filtered to eliminate bacterial aggregates and quantitatively cultured on GC agar to verify the dose. Vaginal mucus from mice was quantitatively cultured on GC-VCNTS agar daily for 12 days, except for infections using 10^5 CFU in which qualitative cultures were performed. Stained vaginal smears were examined under light microscopy to visualize gonococci associated with PMNs during infection and to determine the percentage of PMNs among vaginal cells as described previously (22). Samples from mice inoculated with saline (placebo controls) were evaluated to provide a baseline for measuring the host PMN response to infection, which was defined as the average percentage of PMNs detected in control mice plus one standard deviation. Animal experiments were conducted in the laboratory animal facility at the

FIG. 1. Association and internalization of wild-type or *kat* mutant *N. gonorrhoeae* by murine PMNs. The average number of PMNs with bacteria was determined as described in Materials and Methods, and the data were stratified to show different degrees of uptake or adherence as indicated by the key. Standard error bars are shown. (A) FA1090 (associated); (B) FA1090 (associated and internalized); (C) GP501 associated; (D) GP501 associated and internalized.

Uniformed Services University, which is fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care under a protocol approved by the University's Institutional Animal Care and Use Committee.

Statistical analysis. Data were analyzed by using the standard versions of SPSS and GraphPad Prism 4 for Windows software. PMN killing assays were first analyzed by the nonparametric Mann-Whitney U test, followed by an unpaired *t* test for comparison of the means. An unpaired *t* test was used to analyze the results of the acridine orange-trypan blue staining assay, the duration of the recovery of wild-type or *kat* mutant bacteria from mice, and the number of CFU recovered at individual time points.

RESULTS

Interactions between *N. gonorrhoeae* and murine PMNs. We previously reported that *kat* mutant GP501 was more susceptible to H₂O₂ and intracellular ROS induced by paraquat than the parent strain FA1090 (43). To determine whether catalase renders *N. gonorrhoeae* more susceptible to killing by PMNs in the murine system, we first standardized conditions by which we could examine the association of gonococci with mouse PMNs. We found that opsonization of the bacteria with NMS was required for efficient association and uptake of *N. gonorrhoeae* as measured by acridine orange-trypan blue staining. PMNs associated with NMS-opsonized gonococci were detected at 30 min of incubation, with significant numbers visualized after 60 and 90 min compared to the number of bacteria

seen when HI-NMS was used ($P < 0.05$) (Fig. 1A). Heat treatment significantly reduced bacterial association with PMNs at all time points, which is consistent with bacterial uptake occurring via the PMN C3b receptor. Internalized gonococci were visualized at 90 min of incubation, and intracellular bacteria were detected at 135 min (Fig. 1B). As reported for human PMNs (15), treatment of mouse PMNs with phorbol myristate acetate increased the degree of bacterial association with the cells (data not shown). We observed no difference in the degree of bacterial association or internalization when we compared PMNs from untreated mice and from mice treated with 17 β -estradiol (data not shown). We also assessed the oxidative response of PMNs elicited by peritoneal lavage to *N. gonorrhoeae* using an LDCL assay. PMNs isolated from untreated or estradiol-treated mice exhibited a dose-dependent LDCL response upon exposure to strain FA1090 that was higher than the background signal produced by unstimulated PMNs. Estradiol appeared to enhance the phagocytic oxidative response based on the generation of an LDCL response that was consistently 10-fold higher in magnitude in PMNs from estradiol-treated mice compared to untreated mice (Fig. 2).

Effect of *kat* mutation on susceptibility to killing by mouse PMNs. Upon the demonstration that murine PMNs elicited by

FIG. 2. LDCL response of murine PMNs upon exposure to *N. gonorrhoeae* FA1090. PMNs from normal or 17 β -estradiol-treated female BALB/c mice were incubated with increasing numbers of wild-type gonococci. The results shown are representative of six replicates of each experiment. (A) PMNs from untreated mice; (B) PMNs from estradiol-treated mice.

thioglycolate broth were not prematurely activated, we next measured the susceptibility of wild-type and *kat* mutant (GP501) gonococci to PMN killing. Recovery of viable NMS-opsonized wild-type FA1090 was significantly reduced at 135 min ($P < 0.05$) compared to that of gonococci incubated with HI-NMS and PMNs (Fig. 3A). Catalase-deficient GP501 bacteria, in contrast, were killed more quickly, with a significant decrease in the recovery of *kat* mutant gonococci detected at 90 min ($P < 0.05$). The degree to which the *kat* mutant was killed was also more significant than that of the wild-type strain at 135 min ($P < 0.005$) (Fig. 3B). We detected no difference in the capacity of PMNs from estradiol-treated versus untreated mice to kill wild-type or catalase-deficient *N. gonorrhoeae* (data not shown) despite the observation that PMNs from estradiol-treated mice produce a stronger LDCL response. Estradiol treatment also did not affect the susceptibility of *S. aureus* to killing by murine PMNs. Specifically, using the above conditions, recovery of opsonized staphylococci from PMNs from untreated and estradiol-treated mice after 90 min incubation was 53 and 57% of that recovered from media without PMNs, respectively.

FIG. 3. Opsonophagocytic killing of wild-type or *kat* mutant gonococci. The average number of viable gonococci recovered after incubation of gonococci with PMNs from estradiol-treated mice for 45, 90, or 135 min is shown. The data were analyzed by comparing the recovery of gonococci that were preincubated with NMS ($\square$) versus HI-NMS ($\blacksquare$). Levels of significance are denoted as follows: *, $P < 0.05$; **, $P < 0.005$ (as determined by unpaired Student *t* test). (A) Wild-type FA1090; (B) *kat* mutant GP501. The increased recovery of the *kat* mutant from the HI-NMS at 135 min was not reproducible in two other experiments.

The increased susceptibility of the *kat* mutant to PMN killing could not be explained by differences in association or the uptake by PMNs (compare Fig. 1C and D to Fig. 1A and B). There was no difference in the growth rate of wild-type FA1090 or *kat* mutant GP501 bacteria when cultured aerobically in iron-supplemented broth into the late stationary phase, and the stock cultures used in these studies did not differ in phase-variable phenotypes that might influence the results. Specifically, the degree of piliation, as measured by transformation frequency, and the predominant opacity protein and LOS phenotypes were the same for wild-type and *kat* mutant strains (data not shown). Wild-type and *kat* mutant gonococci induced an LDCL response that was comparable in kinetics and magnitude, although in general the wild-type strain induced a slightly higher response (Fig. 4A). The addition of bovine catalase to the assay reduced the LDCL response to either wild-type or *kat* mutant gonococci by 50%. No inhibition was observed when NaN_3 and HRP were added unless cytochalasin B was also used. These results are consistent with mostly intracellular ROS (Fig. 4B); a predominantly intracel-

FIG. 4. LDCL response of murine PMNs to wild-type or catalase-deficient gonococci. (A) LDCL over time after exposure to FA1090 or GP501 gonococci; (B) percentage of peak LDCL response in samples treated with catalase or HRP and NaN₃ to analyze the location of the ROS (extracellular versus intracellular). The results represent combined data from six different experiments; standard error bars represent variability between experiments. PMNs were from 17 β -estradiol-treated mice and the multiplicity of infection was 10:1.

lular response to *N. gonorrhoeae* was reported for human PMNs (31, 41).

Survival of catalase-deficient gonococci in vivo. To examine the importance of catalase during mucosal infection, we next examined the capacity of mutant GP501 to establish genital tract infection in estradiol-treated mice. Mice were inoculated intravaginally with ca. 10⁶ CFU of wild-type FA1090 or GP501 gonococci, which is the 80% infective dose for strain FA1090 in this model, and vaginal mucus was cultured for 12 consecutive days. In two of six experiments, the duration of infection was significantly shorter for the *kat* mutant than for the wild-type strain ($P < 0.01$); however, in the remaining four experiments, there was no difference (five to eight mice per group). Combined data from the six experiments in which 10⁶ CFU were used showed earlier clearance of the *kat* mutant ($P \leq 0.013$) (Fig. 5). In a single experiment in which 10⁵ CFU were used, there was no significant difference in the duration of infection (averages of 10.5 days [wild type] versus 9 days [*kat* mutant]; six

FIG. 5. Duration of colonization of wild-type versus catalase-deficient gonococci. Mice were inoculated with 10⁶ CFU of FA1090 or GP501 gonococci and cultured for 12 days. The last positive culture day for each individual mouse is indicated by closed (wild type) and open (*kat* mutant) circles. The results shown are combined data from six experiments. The average duration of recovery is shown by the horizontal bar and was significantly different when *kat* mutant and wild-type bacteria were compared ($P \leq 0.013$). The P values for the individual experiments were 0.107, 0.013, 0.54, 0.35, 0.38, and 0.001 (five to eight mice per group).

mice per group). In two of three experiments in which quantitative culture of vaginal mucus was performed, a higher average number of CFU was recovered from mice infected with the wild-type strain compared to that from mice infected with the *kat* mutant (Fig. 6); however, the difference was not significant.

A higher percentage of vaginal PMNs was detected in a subset of mice in each experiment than that observed in placebo controls, and we frequently observed PMNs associated with numerous wild-type or *kat* mutant gonococci (Fig. 7). The *kat* mutant consistently induced a higher percentage of PMNs than the wild-type strain; the reason for this observation is not known. For mice inoculated with strain FA1090, periods of

FIG. 6. Colonization load during infection with wild-type versus catalase-deficient gonococci. Mice were inoculated with 10⁶ CFU of FA1090 or *kat* mutant GP501 gonococci. The average number of viable wild-type FA1090 and *kat* mutant GP501 CFU recovered from vaginal swab suspensions over time. Standard error bars are shown.

FIG. 7. Stained vaginal smears from mice infected with wild-type or *kat* mutant gonococci. (A) Uninfected mouse; (B and D) mouse inoculated with FA1090; (C and E) mouse inoculated with *kat* mutant GP501. Samples were from day 8 of infection. Numerous PMNs with high numbers of wild-type or *kat* mutant gonococci are seen. The images are shown at magnifications of $\times 400$ (A, B, and C) and $\times 1,000$ (D and E).

PMN influx that lasted 2 to 5 days occurred in three of four (75%), three of eight (38%), and one of six (17%) mice; three of three (100%), six of seven (86%), and four of seven (57%) mice inoculated with *kat* mutant GP501 demonstrated periods of increased PMNs (average percentages of mice with vaginal PMN response in three experiments of 43% [wild type] and 81% [mutant]). Both wild-type and *kat* mutant gonococci persisted during periods PMN influx, and gonococci were recovered from mice with as many as 50 to 64% PMNs in vaginal smears. As reported previously (22, 23), we observed mouse-to-mouse variability in the number of gonococci recovered, as well as variability in PMN influx within individual mice over the course of infection. No correlation could be established, however, between the degrees of PMN influx, clearance of infection, or colonization load in either group (Table 1). We also found no evidence of a phenotypic reversion of the *kat* mutation during infection in any of these experiments, as assessed by testing thousands of vaginal isolates from mice infected with mutant GP501 by the bubbling assay.

DISCUSSION

The importance of catalase in the evolution of *N. gonorrhoeae* is supported indirectly by the observation that catalase-deficient gonococci are rare in the laboratory and among clinical isolates (25). Detoxifying enzymes such as catalase may

protect bacteria by directly neutralizing ROS produced by normal respiration, exposure to metal ions, phagocytes, epithelial cells, and H_2O_2 -producing commensal flora as recently reviewed by Seib et al. (40). Here we showed that catalase was not required for infection of the lower genital tract of female mice, at least not when doses equal to or 10-fold lower than the 80% infective dose were used. Combined data from several experiments suggested the *kat* mutant was less able to colonize mice for extended periods of time than the wild-type strain. There was much variability between experiments, however, and no difference was detected in a single experiment that used a lower dose. We conclude that catalase may confer a minor advantage to the survival of strain FA1090 in vivo, which is easily obscured by animal variability. Based on power calculations derived from the data we have, we predict we would need more than 250 mice per group to detect a difference of <3 days duration for the wild-type and *kat* mutant bacteria 95% of the time. The reason for this apparent advantage is not yet known. Although we found the *kat* mutant to be more susceptible to PMN killing in vitro, the recovery of high numbers of catalase-deficient gonococci from mice with a vigorous PMN influx is inconsistent with the likelihood that catalase plays a significant role in gonococcal defense from phagocytes in vivo.

Due to the dependency of the respiratory burst on molecular oxygen, the O_2 tension at the site of infection can be critical for the capacity of PMNs to efficiently kill microbes. The lower female genital tract is a relatively anaerobic environment, as indicated by direct measurements of vaginal O_2 tension (34, 51) and an abundance of anaerobic commensal flora (19). It is not known whether ROS are produced by phagocytes during

TABLE 1. Duration of colonization and periods of PMN influx for individual mice inoculated with *N. gonorrhoeae* FA1090 or *kat* mutant GP501

Inoculum and mouse ^a	No. of days colonized ^b	Period (days) of PMN influx ^c
FA1090		
M1	8	6; 10–12
M2	9	4
M3	10	2, 4
M4	12	1–2; 4
M5	12	2, 4
M6	11	None
M7	12	None
M8	9	2–4; 11
GP501 (<i>kat</i>)		
M9	6	4–8
M10	7	6–12
M11	12	2–4; 12
M12	6	2–5
M13	2	None
M14	2	1–7; 11–12
M15	5	6–8
M16	2	None

^a Mice inoculated with FA1090 were designated M1 through M8; mice inoculated with GP501 were designated M9 to M16.

^b That is, the number of days with positive vaginal culture out of 12 consecutive days tested.

^c For periods of PMN influx that lasted longer than 2 days, the average percentage of vaginal PMNs was 41% (range, 12 to 71%) for the wild-type strain and 50% (range, 10 to 94%) for the *kat* mutant. Inflammation was defined as a higher percentage of PMNs than the baseline percentage as described in Materials and Methods. For this experiment, the baseline was 6% PMNs.

cervical infections or if ROS challenge gonococci in vivo. However, a growing body of work suggests that *N. gonorrhoeae* are strongly resistant to ROS produced by phagocytes, the most definitive evidence being a study by Rest et al. (36), which showed no difference in the killing capacity of PMNs from normal individuals and those with chronic granulomatous disease. Other evidence includes studies on PMN killing under aerobic versus anaerobic conditions (9) and a recent report that showed gonococci replicate within PMNs that are actively producing ROS (41). The basis for gonococcal resistance to PMN killing is not known. At this time we cannot fully rule out that catalase may at least partially protect *N. gonorrhoeae* from ROS produced by phagocytes in the murine genital tract. *N. gonorrhoeae* has a rich layer of antioxidant factors and a functional redundancy of these potentially protective factors may obscure the role of catalase in vivo. In addition to catalase, cytochrome *c* peroxidase (Ccp) (48), nonenzymatic quenching of O_2^- by manganese cations (47), methionine sulfoxide reductase (which repairs oxidatively damaged proteins) (42), a potential thiol-disulfide oxidoreductase (Sco) (38), azurin (53), bacterioferritin (11), and newly described peroxidase-induced genes of unknown function (44) may protect the gonococcus from ROS in vivo. Seib et al. recently reported that a double *kat ccp* mutant and a *kat sco* mutant were not more susceptible to PMN killing than the wild-type strain (39). Although several combinations of other mutations were not tested, these results are consistent with the highly resistant nature of *N. gonorrhoeae* to oxidative defenses of PMNs (9, 36, 41).

Clues from the literature regarding the role of catalase in the pathogenesis of other bacteria vary with the pathogen and the site of infection. Gonococcal catalase belongs to the typical monofunctional group of bacterial catalases (30, 43); among other members of this group, *katA* mutants, but not *katB* or *katE* mutants, of *Pseudomonas aeruginosa* were attenuated in a mouse peritonitis model (28), and an *htkE* catalase mutant of *Haemophilus influenzae* showed reduced survival in rats after intraperitoneal injection. No role for HtkE catalase was detected during *H. influenzae* bloodstream infection after dissemination from an intranasal site, however (5). Similarly, a *katE* mutant of *Brucella melitensis* colonized internal organs as well as the wild-type strain after conjunctival inoculation of pregnant goats; colonization of the fetus was also not impaired (17). With regard to localized mucosal infections, the HtkE catalase of *H. influenzae* did not confer a survival advantage for intranasal colonization (5) and a *Bordetella pertussis katA* mutant was not attenuated for colonization of the mouse respiratory tract (26). The overall consensus of in vivo studies on typical monofunctional catalases suggests that the evolution of catalases in this family often may have been driven by the need to protect against ROS produced by respiration. This conclusion is in contrast to that shown for some members of the catalase-peroxidase family of bacterial catalases, which clearly play a role in pathogenesis (2, 32). Alternatively, and as proposed in each study that detected no role for a monofunctional catalase during infection, a functional redundancy in antioxidant factors may mask the contribution of catalase in pathogenesis.

We did observe that the *kat* mutant used here was more sensitive to killing by murine PMNs in vitro. Attempts to confirm our PMN killing data via genetic complementation with a

wild-type *kat* gene have been frustrating. We believe interpretation of PMN killing assays should also be tempered by recognizing both the lack of complementation data and the fact that many physiological variables play a role in the PMN killing of *N. gonorrhoeae*. As described previously (43), the *kat* mutant used here was only partially complemented for catalase activity using a plasmid shuttle vector. The recombinant catalase expressed by this vector was aberrant in charge or size, as evidenced by an altered mobility on activity gels, and was unable to restore resistance to H_2O_2 . More recently, we integrated a wild-type copy of the *kat* gene into the chromosome of *kat* mutant GP500, a derivative of GP501 (43). Although the resultant strain produced a catalase species that appeared to be similar to wild-type catalase on activity gels, expression was low and unstable.

Seib et al. (39) recently showed no difference in the survival of wild-type or *kat* mutant bacteria in human PMNs using a system in which PMNs are adherent to an extracellular matrix, which may better mimic the interaction with PMNs in vitro. In contrast, we assessed PMN killing by using an assay in which suspensions of PMNs and gonococci were incubated in tubes that were rotated end to end. This method increases contact between bacteria and PMNs in a way that might not mimic infection and maximizes aeration to a level that might be artificially high compared to that available in inflammatory foci. Our inability to correlate clearance of the *kat* mutant with a strong PMN response during infection is in conflict with the observed increased susceptibility of the *kat* mutant to PMN killing in vitro. Therefore, the PMN killing assay we used may not be predictive of events that occur during mucosal infection of this body site. Strain differences may also contribute to different observations, based on recent evidence from our lab that a *kat* mutant in strain MS11 was not attenuated for PMN killing using the tumbling tube assay (53a).

A second objective of our study was to further characterize the usefulness of murine infection in studying gonococcal evasion of innate defenses. Estradiol is known to have immunosuppressive effects. Notably, Kita et al. (27) reported that PMNs from estradiol-treated mice were less able to kill *N. gonorrhoeae* compared to PMNs from untreated or progesterone-treated mice. We found no evidence that estradiol treatment impaired the capacity of PMNs to kill *N. gonorrhoeae* or *S. aureus*. Differences in the estradiol dose and delivery systems used may explain this discrepancy. We also showed that PMNs from estradiol-treated versus normal mice responded similarly to wild-type and *kat* mutant bacteria in terms of the intracellular nature of the respiratory burst and the capacity of murine PMNs to adhere to and take up *N. gonorrhoeae*. Known differences between mouse and human PMNs include lower levels of myeloperoxidase and other enzymes in mouse PMNs (35) and the inability of gonococci to utilize mouse lactoferrin as an iron source, which is contained within PMN granules. Gonococci do utilize the mouse hemoglobin that exudes into the vaginal lumen during periods of inflammation, however, which may help balance any detrimental effects caused to gonococci by the inflammatory response (23). An additional limitation to the murine system is the absence of human carcinoembryonic antigen cellular adhesion molecules on PMNs, which when bound to certain gonococcal opacity proteins results in nonopsonic uptake. The murine system, in contrast,

requires serum for uptake and therefore most likely mimics phagocytic events that occur later in infection when complement components are increased with the onset of inflammation.

In summary, we have shown that catalase is not required for gonococci to survive the innate defenses of the female genital tract by using a surrogate infection model. Comparative studies of mutants in more than one antioxidant factor and the use of mice that are functionally defective in phagocytic killing should help further define the interplay between *N. gonorrhoeae* and phagocytes in vivo.

ACKNOWLEDGMENTS

This study was supported by National Institutes of Health grant number AI042053. A.A.S.-G. was supported by NRSA grant F31 AI10494.

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Editor: J. N. Weiser