Interactions of *Neisseria gonorrhoeae* with Adherent Polymorphonuclear Leukocytes

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Neisseria gonorrhoeae causes severe exudative urethritis. The exudates from infected individuals contain large numbers of polymorphonuclear leukocytes (PMN) with ingested gonococci. The fate of *N. gonorrhoeae* within PMN has been a topic of debate for years. In this study, we examined the interactions of *N. gonorrhoeae* with PMN adherent to surfaces as a system that better models events during clinical disease. Using chemiluminescence to measure reactive oxygen species (ROS), we found that *N. gonorrhoeae* stimulated PMN to produce a respiratory burst. Different kinetics were seen when PMN were stimulated with opsonized zymosan particles. In addition, ROS were produced predominantly inside the PMN in response to gonococci. Laser scanning confocal microscopy and transmission electron microscopy showed that *N. gonorrhoeae* rapidly associated with PMN under these experimental conditions and was internalized. Some gonococci were cleared in the first 30 to 60 min after phagocytosis, but a majority of the population persisted for 6 h after phagocytosis. Quantification of viable organisms showed that a significant portion of the population resisted killing. The viability of this subpopulation remained unchanged for 2 h after phagocytosis. A significant increase of viable gonococci from 1 to 6 h was also observed, suggesting intracellular replication. Four different *N. gonorrhoeae* strains demonstrated the same capacity to resist PMN-mediated killing, whereas *Escherichia coli* was rapidly killed by PMN under the same conditions. Taken together, these findings suggest that a subpopulation of *N. gonorrhoeae* resists killing and replicates within PMN phagosomes in spite of NADPH oxidase activation.

Neisseria gonorrhoeae (gonococcus) is a gram-negative diplococcus that causes the sexually transmitted disease gonorrhea in humans. Infection with *N. gonorrhoeae* can result in an acute urethritis. Urethral exudates from infected individuals show predominantly polymorphonuclear leukocytes (PMN), many with large numbers of ingested gonococci (1, 10, 24, 25). Despite the exuberant and rapid PMN recruitment to the site of infection, persistence of disease for weeks to months indicates that the inflammatory response is ineffective in eradicating organisms and suggests that *N. gonorrhoeae* may possess mechanisms to evade killing by PMN.

PMN are professional phagocytes that migrate to sites of infection, where they ingest microorganisms by phagocytosis (11, 13, 20). PMN kill microorganisms by the combined activity of antimicrobial proteins and reactive oxygen species (ROS). The NADPH oxidase is a multicomponent enzyme responsible for agonist-dependent generation of ROS by PMN (2, 8, 33). The oxidase complex reduces molecular oxygen to form superoxide anion (O_2^-), which is converted into hydrogen peroxide (H_2O_2). Hypochlorous acid (HOCl) is then generated through the activity of myeloperoxidase (MPO) (33). PMN ROS generation, referred to as the respiratory burst, is a primary killing component of PMN antimicrobial activity and represents an important element in innate host defense against microorganisms. The contribution of ROS to PMN antimicrobial action is illustrated by the clinical complications in chronic granuloma-

tous disease, a genetic disorder that results in lack of NADPH oxidase activity (33). Although PMN from chronic granulomatous disease patients exhibit normal migration, phagocytosis, and degranulation, they fail to produce oxidants and thus exhibit ineffective killing of a variety of microorganisms. PMN also possess nonoxidative mechanisms of killing that include an array of antimicrobial proteins and enzymes contained within a variety of cytoplasmic granules that are released into the phagosome upon ingestion (11). The combination of both oxidative and nonoxidative mechanisms allows the PMN to effectively kill a broad range of microorganisms.

The fate of *N. gonorrhoeae* within PMN has been a topic of debate for years. Differences in strains and experimental systems may account for the discrepancies in conclusions regarding the relative importance of oxidants and the fate of *N. gonorrhoeae* within PMN. In the following study, the interactions of *N. gonorrhoeae* with adherent PMN were investigated using a synchronized phagocytosis system to allow control of bacterial association and uptake by PMN. Using this system, we found that a subpopulation of gonococci survived and replicated within PMN over time, results that mirror the clinical aspects of gonococcal disease.

MATERIALS AND METHODS

PMN isolation. Heparinized blood was drawn from healthy volunteers according to a protocol approved by the institutional review board at The University of Iowa. PMN were isolated by dextran sedimentation and Hypaque-Ficoll (Amersham) density gradient separation as previously described (3). Residual erythrocytes were removed by hypotonic lysis, and PMN were resuspended in Hanks balanced salt solution (Gibco) with Ca^{2+} and Mg^{2+} and supplemented with 0.15% dextrose and 1% human serum albumin (HBSS+). Isolation yielded >99% PMN that were >95% viable as measured by trypan blue exclusion.

Bacterial cultures. *N. gonorrhoeae* strains 1291, 1291gfp, FA1090, F62, and PID2 and *Escherichia coli* K-12 (M. A. Apicella personal culture collection) were stored as 20% glycerol stocks at −80°C. *N. gonorrhoeae* strains were pili and opa positive as determined by colony morphology as previously described (17, 39). Strains were passed onto gonococcus (GC) or Luria-Bertani agar plates overnight prior to the experiment and were suspended in GC broth (Difco) and grown to mid-logarithmic phase. Bacterial concentration was determined based on the optical density at 600 nm. Cultures were diluted in HBSS+ containing 1% autologous normal human serum (HBSS+S) prior to the experiment.

Synchronized phagocytosis experiments. Glass coverslips coated with bovine tendon collagen were added to 24-well tissue culture plates and incubated in autologous normal human serum (NHS) for 30 min at 37°C prior to adherence of PMN. Approximately 2×10^6 PMN were added to each well of the 24-well tissue culture plate, centrifuged at $350 \times g$, and allowed to adhere for 30 min at 37°C. Monolayers were washed with 0.9% NaCl to remove nonadherent PMN. Medium was replaced with HBSS+S, and cells were kept at 10°C. To quantify numbers of adherent PMN, cells were released by trypsin-EDTA treatment and counted using a hemacytometer. Bacteria were added to PMN at a multiplicity of infection (MOI) of 1:1 or 10:1 and centrifuged at $800 \times g$, 10°C, to allow the bacteria to bind to PMN but not be ingested. Supernatants were removed and replaced with HBSS+S warmed to 37°C to initiate phagocytosis. This procedure was used for all the experiments outlined below.

Viable counts of intracellular bacteria. PMN were challenged using the synchronized phagocytosis protocol described above. Supernatants were removed and replaced with HBSS+S warmed to 37°C for 2 min to initiate phagocytosis. Monolayers were then washed three times with 0.9% NaCl to remove extracellular bacteria. PMN were placed in HBSS+S and incubated in a 37°C water bath for the indicated time points. This point in the assay was designated as zero time (T_0). Monolayers were treated with 1% saponin for 10 min at 37°C and scraped to lyse the cells. Clumping of gonococci was occasionally seen by microscopy, especially during early time points in the assay. To disperse the clumps and obtain accurate CFU counts, lysates were vortexed for 30 to 60 s in glass tubes. Lysates were then serially diluted and plated to enumerate CFU. Data are expressed as CFU at respective time points relative to the CFU at T_0.

Chemiluminescence. Opsonized zymosan (OPZ) stimulates a robust respiratory burst in PMN and was used a positive control for the chemiluminescence experiments. Zymosan A (ICN) was opsonized with 10% NHS for 30 min at 37°C. PMN were adhered to collagen-coated coverslips and stimulated with OPZ or *N. gonorrhoeae* strain 1291 in the presence of 100 μM lucigenin, 1 μM luminol, or 50 μM isoluminol (Sigma). Phagocytosis was synchronized by centrifugation at $800 \times g$, 10°C. Horseradish peroxidase (8 U/ml) was added to wells tested for isoluminol chemiluminescence. Chemiluminescence was measured using a Wallac Victor2 luminometer (Perkin-Elmer) with readings taken every 2 min for 1 h. The different chemiluminescence substrates were chosen to determine the type of ROS products generated and the location of their release. Lucigenin and luminol detect both intracellular and extracellular ROS production, but they differ in the substrates with which they react. Lucigenin reacts with superoxide (products of the NADPH oxidase complex) (14), whereas luminol chemiluminescence depends on the activity of MPO and reacts with hydroxyl radical and HOCl (7, 38). Isoluminol reacts with the same products as luminol but lacks the ability to penetrate the cell membrane and thus measures extracellular ROS production (21).

Microscopy. (i) LSCM. PMN were challenged with *N. gonorrhoeae* strain 1291gfp by using the synchronized phagocytosis protocol, incubated for the indicated times, washed to remove unbound gonococci, and fixed with 2% paraformaldehyde at the desired time points. Coverslips were mounted onto glass slides and viewed using a Zeiss 510 confocal microscope. PMN were visualized using differential interference contrast (DIC) light microscopy. Green fluorescent protein (GFP)-labeled bacteria were viewed using the fluorescein isothiocyanate (FITC) fluorescence channel. Images were taken as optical sections from apical to basolateral cell surfaces at approximately 0.5-μm intervals. Zeiss LSM software was used to compile optical sections into a single image. Cell association was determined by merging FITC and DIC channels. The numbers of GFP-positive gonococci associated with PMN were determined by visual counts of confocal images. PMN were challenged with gonococci at an MOI of 10:1, and phagocytosis was synchronized as described above. At each respective time point, the PMN monolayers were washed to remove nonassociated gonococci and coverslips were processed for laser scanning confocal microscopy (LSCM) analysis. Approximately 100 PMN were counted at each time point and grouped in the following categories: PMN with 0, 1 to 9, or 10 or more gonococci/cell. This method was used to assess the kinetics of phagocytosis and to understand the dynamics of the gonococcal population within PMN over time.

(ii) SEM. PMN were challenged using the synchronized phagocytosis protocol and fixed with 2.5% glutaraldehyde at the desired time points. Coverslips were processed for scanning electron microscopy (SEM) analysis by osmium fixation followed by ethanol dehydration and were mounted onto aluminum stubs. Samples were examined using a Hitachi S-4000 scanning electron microscope.

(iii) TEM. PMN were challenged using the synchronized phagocytosis protocol for the desired time points and released with trypsin-EDTA. PMN were pelleted by centrifugation at $1,000 \times g$ and fixed with 4% glutaraldehyde. Samples were processed for transmission electron microscopy (TEM) by osmium fixation followed by staining with 2.5% uranyl acetate and ethanol dehydration. Cell pellets were embedded in eponate 12 (Ted Pella, Redding, Calif.), and thin sections were cut using a Reichert Ultracut E ultramicrotome (Vienna, Austria). Sections were placed onto formvar grids and examined using a Hitachi H-7000 transmission electron microscope.

Statistical analysis. Data are the mean of at least three replicates and are presented as standard error of the mean values. Means were compared using paired *t* tests with a confidence interval at $P < 0.05$. Statistically significant values are indicated. Statistical analysis was performed using the GraphPad Prism 4 software package.

RESULTS

***N. gonorrhoeae* stimulated respiratory burst in adherent PMN.** Chemiluminescence studies were performed to determine if adherent PMN were capable of producing a respiratory burst in response to gonococci (Fig. 1). PMN were stimulated with either OPZ or a dose range of *N. gonorrhoeae* strain 1291 in the presence of lucigenin, luminol, or isoluminol. In the presence of lucigenin (Fig. 1A) or luminol (Fig. 1B), PMN produced significant chemiluminescence when stimulated with 10 or more gonococci/PMN. The amount of chemiluminescence produced in response to *N. gonorrhoeae* was dose dependent. Opsonization of gonococci had no effect on the chemiluminescence response (data not shown). Stimulation of both lucigenin and luminol chemiluminescence by gonococci suggests that both products of the NADPH oxidase (O_2) and MPO (hydroxyl radical, HOCl) are released. PMN responded very rapidly to stimulation with OPZ, with maximal chemiluminescence detected at approximately 10 min. In contrast, *N. gonorrhoeae*-challenged PMN achieved maximum chemiluminescence at approximately 20 min. The differences in kinetics suggest different mechanisms of activation. Isoluminol (Fig. 1C) was used to determine the amount of extracellular ROS produced. PMN challenged with gonococci produced chemiluminescence in the presence of isoluminol, but with 1/10 the amplitude compared to lucigenin and luminol experiments. These findings suggest that the majority of ROS produced in response to gonococci was primarily intracellular with some release into the extracellular environment.

***N. gonorrhoeae* cells associated with PMN and were persistent as a population over time.** LSCM and SEM were performed to examine association of gonococci with PMN (Fig. 2A and B). LSCM illustrated that gonococci expressing GFP were within the perimeter of the PMN membrane visualized with the light channel and there were very few GFP-expressing bacteria residing in the extracellular spaces (Fig. 2A). SEM also showed that there were no gonococci within the extracellular spaces (Fig. 2B). Removal of nonassociated bacteria by washes was essential to accurately quantify numbers of intracellular bacteria. Cell-associated gonococci were counted at various time points to assess the dynamics of the bacterial population over time (Fig. 2C). At 0 min, 75.6% of PMN counted had 0 associated gonococci, suggesting that gonococci
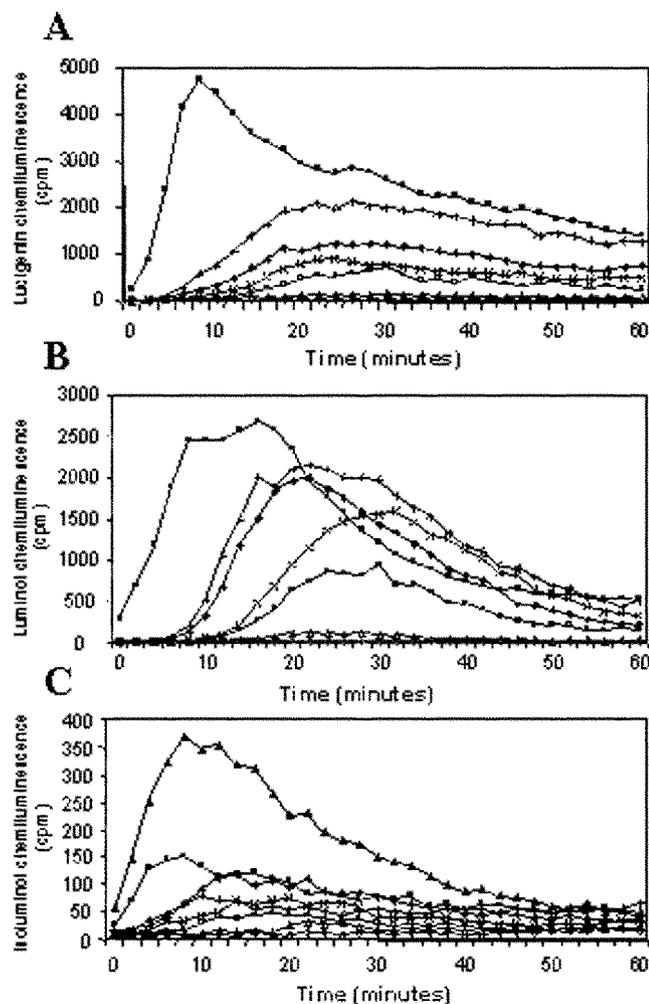
FIG. 1. Chemiluminescence. Adherent PMN were stimulated in the presence of lucigenin (A), luminol (B), or isoluminol (C) with 1:1 OPZ (■) or the following dose ranges of *N. gonorrhoeae* strain 1291: 1:1 (△), 10:1 (□), 20:1 (×), 50:1 (♦), 100:1 (+). Unstimulated, resting cells (◇) were used as a negative control. For isoluminol chemiluminescence, 5:1 OPZ (▲) was used to stimulate a larger response. Chemiluminescence readings were taken every 2 min for 1 h. Data are the result of a single representative experiment, with each value presented as the mean of three replicates.
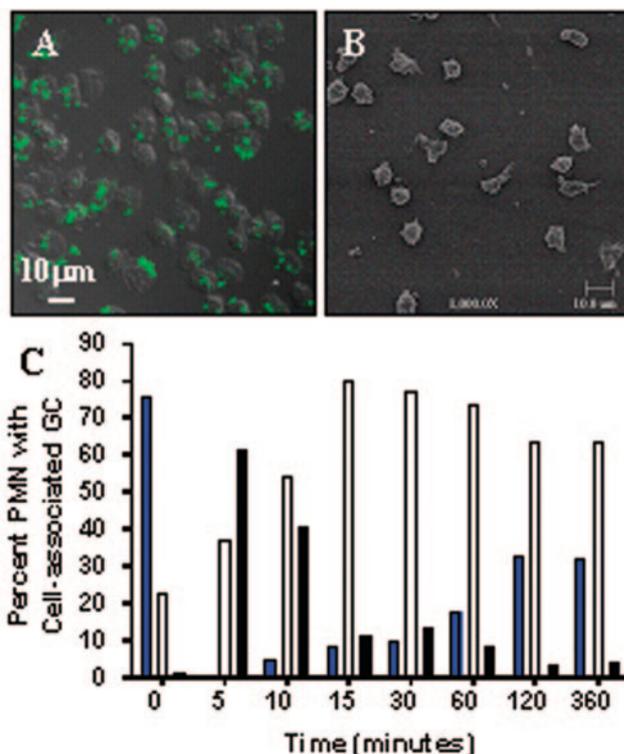

FIG. 2. *N. gonorrhoeae* associated with PMN. (A and B) PMN were challenged with *N. gonorrhoeae* strain 1291gfp at an MOI of 10:1 for 15 min and examined by LSCM (A) and SEM (B). The LSCM image was viewed at 630× magnification, with GFP-containing bacteria seen using the FITC fluorescence channel and PMN viewed by DIC. SEM images were viewed at 1,000× magnification. (C) PMN were challenged with *N. gonorrhoeae* strain 1291gfp at an MOI of 10:1 for the indicated times, washed, fixed with 2% paraformaldehyde, and examined by LSCM. Cell-associated gonococci were counted, and PMN were grouped according to the numbers of associated gonococci: 0 gonococci/PMN (blue), 1 to 9 gonococci/PMN (white), or ≥10 gonococci/PMN (black). Values are the percentages of total PMN counted at each time point.

were washed away before tight association with PMN could occur. However, after 5 min >99% of PMN had associated gonococci, with 61.8% containing 10 or more gonococci/PMN. These data suggest that phagocytosis in this system was synchronous and controlled by the experimental methods. As time progressed, the numbers of PMN with 10 or more gonococci decreased, whereas those with no gonococci increased. After 2 h, the population of cell-associated gonococci remained stable, with the majority of PMN containing 1 to 9 gonococci/PMN throughout the 6-h experiment. Slight increases in the numbers of PMN containing either 1 to 9 or 10 or more gonococci were noticeable from 2 to 6 h. PMN remained viable throughout the experiment, and monolayers remained unchanged (data not shown). Since there was no change in the PMN population, these data suggest that at the early time

points after phagocytosis gonococci were cleared, but a subpopulation remained persistent over time.

***N. gonorrhoeae* was internalized by PMN.** TEM was used to determine if *N. gonorrhoeae* was internalized by PMN in this system (Fig. 3). Gonococci were found inside PMN and within defined phagosomes. The phagosomes were large and loose and often contained numerous gonococci. Two types of gonococci were seen within phagosomes: gonococci that appeared intact and undamaged with dark cytoplasms and gonococci with faint cytoplasms that appeared to be damaged. Gonococci that appeared to be intact and with dark cytoplasms were found at all time points during the 6-h experiment, suggesting that the intracellular population was persistent and was not cleared.

***N. gonorrhoeae* survived killing and replicated within PMN.** To assess gonococci viability within PMN, cells were lysed and CFU were quantified (Fig. 4A). *E. coli* was used as a control for PMN killing and was effectively killed, with only 3.11% ± 0.37% viable at 30 min, 1.83% ± 0.36% at 60 min, 1.73% ± 0.37% at 90 min, and 1.37% ± 0.08% at 120 min. In contrast, a much larger subpopulation of gonococci resisted PMN kill-
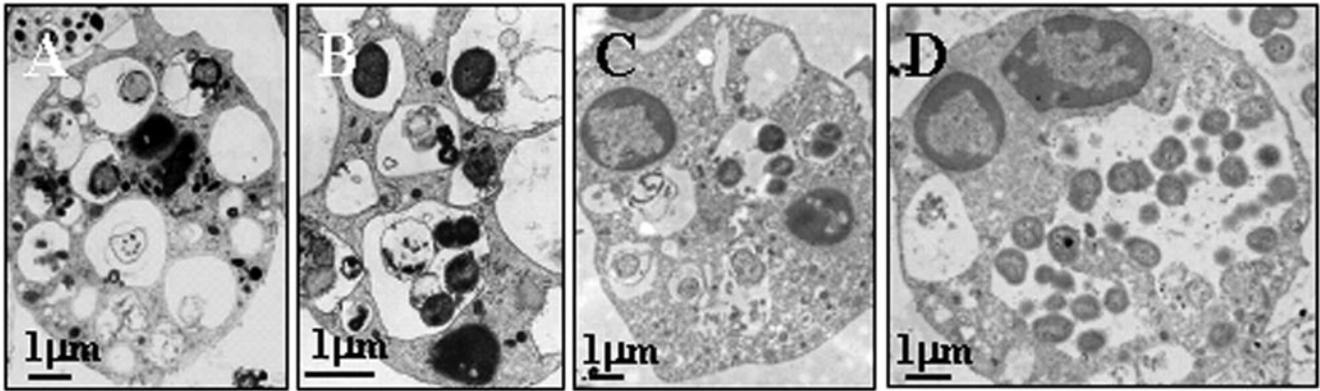
FIG. 3. *N. gonorrhoeae* was internalized by PMN. PMN were challenged with *N. gonorrhoeae* strain 1291 at an MOI of 10:1 and processed for TEM. PMN were challenged for 1 h (A; 8,000×), 2 h (B; 12,000×), 4 h (C; 8,000×), or 6 h (D; 7,000×). Gonococci appear within PMN phagosomes.

ing. In the initial 30 min after phagocytosis, 58.34% ± 9.26% of ingested gonococci were viable. The numbers of gonococci remained stable over the next 2 h, with 75.00% ± 18.31% viable at 60 min, 77.08% ± 3.76% at 90 min, and 80.21% ± 15.34% at 120 min. To determine if gonococci replicate within PMN, cells were challenged with *N. gonorrhoeae* strain 1291 for 1 and 6 h and lysed to quantify intracellular CFU (Fig. 4B). The numbers of intracellular gonococci increased from 83.12% ± 12.15% viable at 1 h to 495.80% ± 47.71% at 6 h. The difference in viable gonococci at 1 and 6 h was statistically significant ($P = 0.0196$). *N. gonorrhoeae* was not able to grow in the experimental medium (data not shown), and so increasing numbers during phagocytosis experiments were indicative of intracellular replication. Since *N. gonorrhoeae* exhibits a high degree of variability of surface structures and virulence factors, we tested three separate strains for comparative analysis (Fig. 5). Although variations in percentages of surviving gonococci were apparent, all of the strains tested exhibited the same capacity to resist killing during the 2-h experiment. These results suggest that *N. gonorrhoeae* survived within PMN and replicated in the intracellular PMN environment.

DISCUSSION

The interaction of *N. gonorrhoeae* with PMN is an important aspect of gonococcal disease that remains unclear. During gonococcal infection, PMN migrate into the urethral lumen and attach to the cells and extracellular matrix present in the surrounding environment. During extravasion from the blood and attachment to surfaces, PMN are activated and thus are phenotypically different from resting PMN in circulation (11, 13). For example, nonadherent PMN are unresponsive to recombinant tumor necrosis factor alpha (rTNF-α) and rTNF-β, but when plated onto surfaces coated with serum, extracellular matrix proteins, or human umbilical vein endothelial cells adherent PMN respond to rTNFs with a robust respiratory burst (23). PMN also exhibit increased phagocytosis after transmigration across cell monolayers (16). These findings demonstrate that PMN adherent to biological surfaces are more responsive than nonadherent PMN.

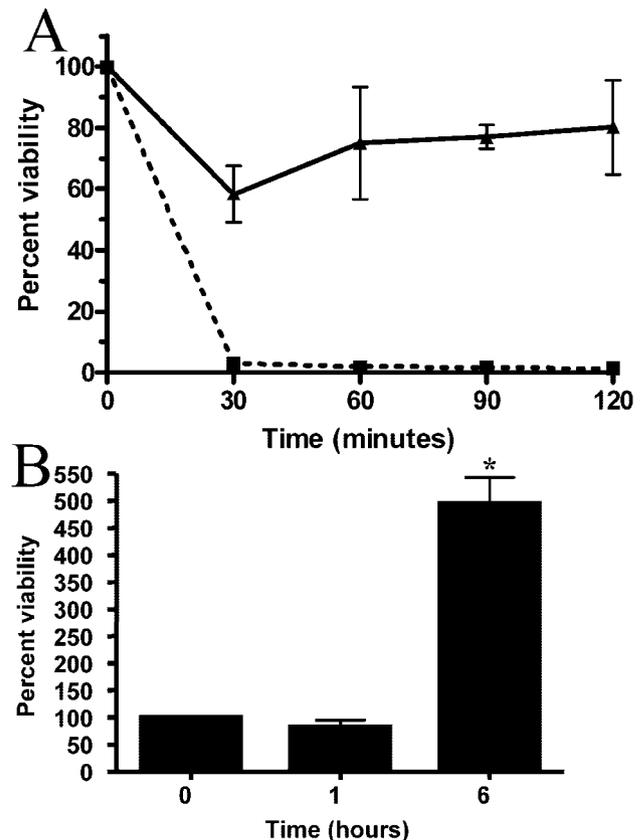Despite the large volume of work addressing PMN-gonococ-

FIG. 4. *N. gonorrhoeae* survived killing and replicated within PMN. (A) PMN were challenged with *N. gonorrhoeae* strain 1291 (▲) or *E. coli* (■) for 2 h at an MOI of 1:1. PMN were lysed at 30-min intervals, serial diluted, and plated for CFU counts. Results are shown as the percentage of viable bacteria present at T_0. Data are the result of a single representative experiment, and values are presented as the mean of three replicates with the standard error of the mean. (B) PMN were challenged with *N. gonorrhoeae* 1291 at an MOI of 1:1 for 1 and 6 h and lysed to quantify CFU. Results are shown as the percentage of viable bacteria at T_0. Data are the result of a single representative experiment, with values presented as the mean of three replicates and the standard error of the mean. Differences were statistically significant ($P = 0.0196$).
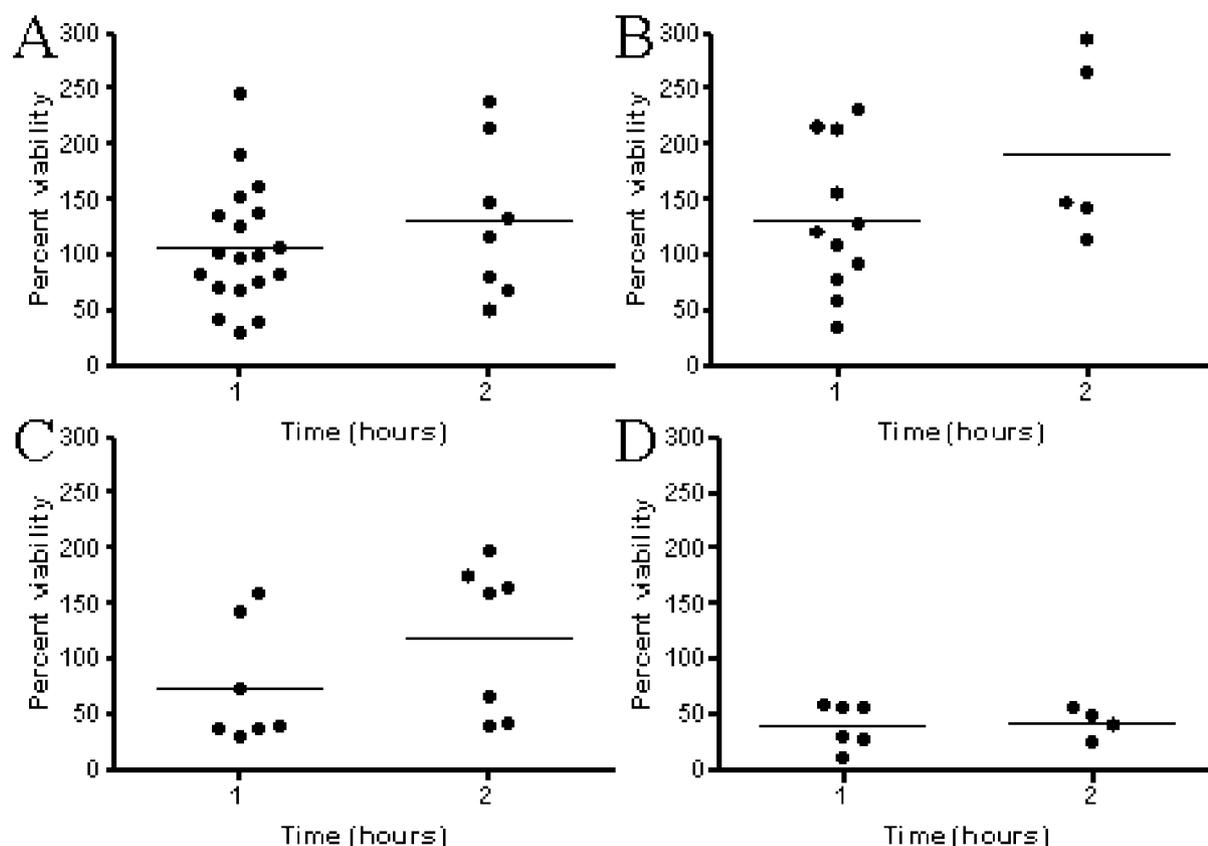
FIG. 5. *N. gonorrhoeae* strain comparison. PMN were challenged with *N. gonorrhoeae* strains 1291 (A), FA1090 (B), F62 (C), or PID2 (D) for 1 and 2 h at an MOI of 1:1. PMN were lysed to quantify CFU. Results are shown as the percentage of viable bacteria at T_0. Each data point is the mean of three replicates performed on individual days.

cus interactions, the fate of *N. gonorrhoeae* within PMN remains unclear. Most of the studies addressing the interactions of gonococci with PMN have used experimental systems with nonadherent PMN. Several studies have concluded that PMN effectively kill gonococci (12, 18, 19, 30–32, 40, 45), but a number of reports have demonstrated that *N. gonorrhoeae* survives killing and may replicate within PMN (4–6, 26–29, 43, 44, 46, 47). We developed a system to examine the interactions of *N. gonorrhoeae* with adherent PMN, reasoning that these PMN more faithfully mimic the PMN recruited into the infected urethra. Isolated PMN were adhered to glass coverslips coated with collagen, a major extracellular matrix protein, and autologous NHS to mimic in vivo conditions. In chemiluminescence studies unstimulated, resting PMN did not produce a respiratory burst, whereas PMN stimulated with OPZ particles produced a respiratory burst as expected (Fig. 1). These findings demonstrate that the adherent PMN responded as expected when challenged with a known stimulus. The lack of chemiluminescence from resting PMN suggests that the experimental conditions did not artificially activate the cells. When exposed to a dose range of *N. gonorrhoeae*, PMN produced a respiratory burst in the presence of both lucigenin and luminol in a dose-dependent manner (Fig. 1A and B). This suggested that products of both NADPH oxidase (O_2^-) and MPO (hydroxyl radical, HOCl) were released in response to gonococci. The kinetics of the respiratory burst elicited by gonococci at all doses were delayed compared to the response to OPZ, suggesting that the mechanisms of activation may differ for the two agonists. Chemiluminescence studies using isoluminol as the substrate were performed to determine the amount of extracellular ROS released (Fig. 1C). Isoluminol chemiluminescence was produced from PMN stimulated with OPZ particles, but only a small amount of isoluminol chemiluminescence was detected in response to gonococci. Comparison of chemiluminescence experiments with the three different substrates suggests that PMN produced primarily intracellular ROS in response to *N. gonorrhoeae*, with only a small amount of ROS released into the extracellular environment. These results are consistent with previous findings that have demonstrated gonococci produce a luminol-dependent respiratory burst in PMN that is predominantly intracellular (12, 22, 31, 45).

Examination of PMN in this system by microscopy showed that the monolayers were not confluent, with variable amounts of space between cells. Increased numbers of PMN added to the system did not result in increased adherence or confluency. With large amounts of space between cells, the concerns of eliminating extracellular bacteria were enhanced. Treatment of cell monolayers with antibiotics to remove nonassociated bacteria was not possible because the antibiotic penetrated the PMN membrane and killed intracellular organisms, a phenomenon previously reported in other phagocytosis systems (9, 15).

To remove nonassociated bacteria we employed systematic washes. LSCM and SEM analyses showed that the washes effectively removed nonassociated bacteria (Fig. 2A and B). Some nonassociated bacteria were occasionally seen, but these were relatively few in comparison to intracellular organisms and gonococci were incapable of replicating in the experimental media. To examine the kinetics of phagocytosis and to monitor the gonococcal population, the numbers of PMN-associated gonococci were determined using LSCM to count GFP-positive gonococci. These experiments showed 75.6% of PMN had no associated gonococci at 0 min. At this time point monolayers were immediately washed after centrifugation, removing most gonococci before they could tightly associate with PMN. However, after 5 min 61.8% of PMN had 10 or more gonococci, indicating rapid association. These data suggest that phagocytosis in this system was synchronous and directed by the experimental methods. PMN with 10 or more gonococci decreased from 61.8% at 5 min to 8.7% at 60 min, while those with 0 associated gonococci increased from 0.8% at 5 min to 17.4% at 60 min. After 60 min the population remained stable until 6 h. PMN challenged with gonococci remained viable over the course of the experiment, and the monolayers remained unchanged over the course of the experiment (data not shown). Since the PMN did not lift off from the coverslips during the experiment, these findings suggest that gonococci were cleared during the first hour after phagocytosis but a subpopulation remained persistent. The presence of serum had no effect on the association of gonococci with PMN (data not shown). Similar studies performed with GFP-expressing *E. coli* demonstrated rapid loss of GFP-positive organisms within minutes after phagocytosis (data not shown). TEM of PMN challenged with *N. gonorrhoeae* showed that cell-associated gonococci were internalized and contained within large phagosomes (Fig. 3). TEM of PMN from our system resembled previously published TEM of PMN from urethral exudates taken from men with gonorrhea (1, 10, 24, 25). Phagosomes often contained several gonococci, some intact and undamaged and others that were damaged and lacked a distinct cytoplasm. The undamaged gonococci persisted throughout the 6-h experiment. These images along with counts of cell-associated gonococci by LSCM (Fig. 2C) add further support to the hypothesis that although some gonococci were initially killed and cleared, a subpopulation remained persistent over time.

Intracellular gonococci were recovered from lysed PMN to determine if intracellular GC seen in microscopy experiments were viable (Fig. 4). Intracellular numbers of *N. gonorrhoeae* strain 1291 decreased to 58.3% in the first 30 min after phagocytosis, indicating that a subpopulation was killed. However, the remaining subpopulation persisted for up to 2 h. *E. coli* was tested as a control over the same time period and under the same conditions, and 99% were rapidly killed by PMN in this system, demonstrating that the adherent PMN possessed effective bactericidal mechanisms. However, gonococci resisted killing and persisted inside PMN. The presence of serum in the experimental medium had no effect on the phagocytosis or intracellular viability of *N. gonorrhoeae* but was important for efficient killing of *E. coli*. The percentages of viable gonococci were calculated relative to CFU at T_0. Accurate quantification of phagocytosis was a limitation of the experimental system. LSCM studies demonstrated that >99% of PMN contained associated GC after 5 min, indicating efficient uptake. However, only 3.27% of the input CFU were recovered at T_0 (data not shown). This likely results from the combination of large numbers of nonassociated GC that did not associate with PMN and were washed away during the assay, as well as rapid killing in the short time periods after phagocytosis. Therefore, the CFU recovered at T_0 were likely an underestimate of actual phagocytosis, but calculation of percent viability relative to T_0 was determined to be a more accurate assessment of viability than calculations relative to the initial inoculum. The important point to consider is not the actual percentages but that a subpopulation resisted killing and remained persistent. Viable counts were performed on PMN challenged with gonococci over 6 h, with an increase of approximately sixfold from 1 to 6 h (Fig. 4B). Analysis of TEM samples at 6 h showed that PMN remained intact and were undamaged despite the increasing numbers of intracellular gonococci. These results suggest that the subpopulation of gonococci that resisted killing were able to replicate within PMN over time. We tested three different strains of *N. gonorrhoeae* (FA1090, F62, and PID2) for comparison to strain 1291 (Fig. 5). Each of these strains resisted killing and persisted during the 2-h experiment. Taken together, these results suggest that although killing of *N. gonorrhoeae* occurred, a subpopulation remained persistent and replicated within PMN over time.

The fate of *N. gonorrhoeae* within PMN has been a focus of study for many years. The work presented in this study demonstrates that *N. gonorrhoeae* resists killing of adherent PMN and replicates in the intracellular environment. These results also suggest that the oxidative killing mechanisms are ineffective against gonococci, since gonococci survived in the presence of intracellular release of ROS. This is consistent with previous reports that oxidative mechanisms are ineffective against *N. gonorrhoeae* (4, 31). Several studies have demonstrated that gonococci possess an array of factors important for resistance to ROS (34–37, 41, 42). The ability of gonococci to resist killing by adherent PMN is important, because PMN are most responsive when adherent to surfaces (23) and exhibit increased phagocytosis after transmigration (16). Since the PMN present in urethral exudates have migrated from the blood during the inflammatory process, our findings suggest that *N. gonorrhoeae* is able to resist PMN killing within the host. Several studies have suggested *N. gonorrhoeae* survives within PMN (5, 6, 26–29, 43, 44, 46, 47). Our results also agree with previous findings examining the interactions of gonococci with PMN monolayers (4). The ability of *N. gonorrhoeae* to resist killing and replicate within PMN is important in the pathogenesis of gonorrhea. Large numbers of PMN are present during gonococcal urethritis, and many of these PMN contain several ingested gonococci. The work presented here suggests that these ingested gonococci remain viable and replicate in PMN phagosomes with time. This helps to explain why gonorrhea can persist for weeks to months despite the large numbers of PMN present at the infection site. The concept of ingested *N. gonorrhoeae* replicating within PMN also presents an attractive model for how gonococcal infection may propagate and be transmitted between sexual partners. Future work needs to address how *N. gonorrhoeae* virulence factors modulate PMN functions to resist killing as well as the effects of intracellular replication on PMN viability.

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Editor: J. N. Weiser