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Stimulation of Human Neutrophil Oxidative Metabolism by Nonopsonized *Neisseria gonorrhoeae*

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Nonopsonized gonococci possessing opacity-associated (Opa; previously PII) outer membrane proteins stimulate neutrophils to undergo a vigorous oxidative response when measured by luminol-dependent chemiluminescence (LDCL). In these studies, we characterized the mechanism of this stimulation. No gonococci that we tested induced measurable release of neutrophil superoxide anion (O_2^-) or hydrogen peroxide (H_2O_2) as measured by reduction of cytochrome *c* or the oxidation of scopoletin, respectively. Neutrophils pretreated with gonococci and then exposed to phorbol myristate acetate, the chemotactic peptide formylmethionylleucylphenylalanine, or opsonized zymosan released levels of neutrophil O_2^- and H_2O_2 comparable to controls, indicating that gonococci were not preventing or inhibiting neutrophil O_2^- or H_2O_2 release. To ascertain a possible explanation for these seemingly contradictory observations (i.e., induction of LDCL, but no release of O_2^- or H_2O_2), we further characterized the ability of Opa$^+$ gonococci to stimulate LDCL. By using 1 mM azide and 4 U of horseradish peroxidase to monitor extracellular LDCL selectively and 2,000 U of catalase to monitor intracellular LDCL selectively, we determined that >80% of total gonococcus-induced neutrophil LDCL occurred intracellularly. In addition, neutrophils stimulated with Opa$^+$ gonococci showed a marked increase in O_2 uptake and hexose monophosphate shunt activity. We conclude that *Neisseria gonorrhoeae* induces neutrophil oxidative metabolism without causing release of detectable amounts of reactive oxygen intermediates into the surrounding milieu. The gonococcus apparently directs oxidase assembly and activity to the phagolysosomal membrane. This could be a mechanism by which extracellular gonococci persist for extended periods in vivo in the presence of high concentrations of neutrophils.

Neisseria gonorrhoeae is an obligate human pathogen that can infect diverse sites within the human host (19, 22, 23, 44, 48, 49). Gonorrhea, the disease caused by *N. gonorrhoeae*, is characterized by a localized purulent urethral or cervical discharge composed of numerous phagocytic cells consisting primarily of neutrophils (40). However, only some of these neutrophils appear to be associated with gonococci; some neutrophils are often packed with intact, seemingly viable intracellular bacteria, while others have gonococci bound to their surfaces (40). Thus, neutrophils may be unable to kill gonococci effectively during early infection. If this were true, subversion of neutrophil killing by gonococci could be considered a virulence factor that can exacerbate the severity and duration of infection. Gonococci can also cause asymptomatic infections with minimal neutrophil involvement (40). This suggests that gonococci have evolved mechanisms by which they can evade the host's defense system and have developed ways to survive these defense systems once encountered.

Much work has focused on identifying and characterizing gonococcal outer membrane components involved in attachment of gonococci to various cell types (5, 15, 18, 23, 29–33, 41, 42, 44, 47). Outer membrane constituents that have received the most attention include pili (19, 23, 31, 33, 44, 47) and the family of opacity-associated (Opa; previously PII) outer membrane proteins (15, 16, 18, 24, 34, 35, 42, 44, 47). Gonococci possessing pili or Opa proteins adhere more avidly to several human cells, including epithelial cells, than do gonococci lacking pili or Opa (23, 24, 33, 40, 44, 48, 49). On the other hand, gonococci possessing most Opa proteins adhere much more avidly to human neutrophils than do Opa$^-$ gonococci (15, 16, 18, 23, 34, 47) regardless of piliation (5, 40, 44, 47). More recently, a 36,000-Da outer membrane protein that recognizes specific glycolipids found in cytoplasmic membranes of mammalian epithelial cells has also been identified (30, 32). This adhesin is present regardless of gonococcal piliation or Opa content and may function in concert with other outer membrane components to facilitate gonococcal adherence to host cells.

Since gonococci possessing most, but not all, Opa proteins adhere to neutrophils and induce neutrophil luminol-dependent chemiluminescence (LDCL), it was concluded that LDCL was a result of stimulation of neutrophil oxidative metabolism by gonococci (15, 18, 34, 47). We have determined that inhibition of binding of Opa$^+$ gonococci to neutrophils by purified Opa proteins also inhibits gonococci from stimulating LDCL, suggesting that Opa-mediated adherence must take place before induction of LDCL (29a).

In this report, we investigated the ability of Opa$^+$ gonococci to induce neutrophil oxygen metabolism. Surprisingly, in spite of an active LDCL, gonococci did not induce the release of either superoxide anion (O_2^-) or hydrogen peroxide (H_2O_2) from human neutrophils; rather, most of the LDCL was intracellular, suggesting that LDCL was a result of phagocytosis followed by phagosome-lysosome fusion, not generalized plasma membrane-lysosome fusion. This could be a mechanism by which the gonococcus causes prolonged infections in untreated gonorrhea.

MATERIALS AND METHODS

Preparation of bacteria. Nonpiliated Opa4 or Opa$^-$ variants of *N. gonorrhoeae* F62 were maintained by daily

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passage on GC agar base (Difco Laboratories, Detroit, Mich.) with added growth supplements as described previously (34, 36). Colony type was identified by criteria established by Kellogg et al. (22), and Swanson (43), and the Opa protein content of the Opa variants was checked regularly by electrophoresis through polyacrylamide gels (15, 27). For daily use, gonococci were grown to mid-log phase in GC broth (GC base without agar) with added supplements at 37°C for 3 to 4 h in a shaking water bath, washed once, resuspended to 2×10^8 to 4×10^8 CFU/ml in phosphate-buffered saline containing 0.1% gelatin (PBSG; pH 7.4), and held at room temperature until used (34, 36).

Neutrophil purification. Human neutrophils were purified from freshly heparinized venous blood by a single centrifugation through Ficoll-Hypaque as described by Ferrante and Thong (17). Erythrocytes were lysed once for 15 s with cold water. Neutrophil suspensions were ≥94% pure as determined by Wright's stain and ≥99% viable as determined by exclusion of 0.25% trypan blue in saline at room temperature. Purified neutrophils were suspended to 1×10^7 to 2×10^7/ml in PBSG and kept on ice until use.

Measurement of neutrophil superoxide release. Superoxide release was measured by monitoring superoxide dismutase (SOD)-inhibitable ferricytochrome *c* reduction by stimulated neutrophils (4). Neutrophils (1×10^6 to 2×10^6/ml in PBSG) were incubated at room temperature in the presence of 1 mg of cytochrome *c* (horse heart, type VI; Sigma Chemical Co., St. Louis, Mo.) per ml with or without 10 μg of SOD per ml. The appropriate stimulus was added, and the increase in A_{550} was monitored continuously in a Perkin-Elmer Lambda 3B dual-beam spectrophotometer. Stimuli used for this work were (i) gonococci (10^8/ml), (ii) phorbol myristate acetate (PMA; 10 ng/ml), (iii) opsonized zymosan (OZ; 150 μg/ml), or (iv) formylmethionylleucylphenylalanine (5×10^{-7} M) in the presence of cytochalasin B (5 μg/ml). Results are expressed as [(A_{550} without SOD) − (A_{550} with SOD)]/min, as A_{550}, or as nanomoles of O_2^- per 10^6 neutrophils (nanomoles of O_2^- per 10^6 polymorphonuclear leukocytes [PMNs]), as determined by using a millimolar extinction coefficient for cytochrome *c* of 29.5.

Measurement of neutrophil hydrogen peroxide release. The rate of neutrophil H_2O_2 release was measured as the reduction of fluorescence of scopoletin at 460 nm with a 350-nm excitation wavelength (37). Briefly, 4 nmol of scopoletin, 22 pmol of horseradish peroxidase (HRP), and 2×10^6 to 3×10^6 neutrophils in 1 ml of PBSG were incubated for 5 min at 37°C before addition of 10 ng of PMA (100-μg/ml stock in dimethyl sulfoxide) or 10^8 gonococci in PBSG. In some experiments, a combination of these agents was used (see below). At various times thereafter, 1.5-ml aliquots were removed and centrifuged at 6,000 × *g* for 4 min at room temperature to effectively stop the reaction. Supernatants were monitored immediately for fluorescence (or were put on ice until read) in an Amino SPF-125 spectrofluorometer (Aminco, Silver Spring, Md.). Results are expressed as percent relative emission intensity of neutrophils incubated with no stimulus, or as nanomoles of H_2O_2 per 10^6 neutrophils (PMNs), determined from a standard curve with reagent H_2O_2.

Measurement of neutrophil CL. Luminol- and lucigenin-enhanced chemiluminescence (CL) (46) was measured in polyethylene vials containing neutrophils (1×10^6 to 2×10^6), 10^{-5} M luminol (10^{-3} M stock in dimethyl sulfoxide), or 10^{-5} M lucigenin (5×10^{-3} M stock in deionized water) and gonococci in a final volume of 1 ml of PBSG, as described previously (16, 28). No serum was present in these assays. In some cases, OZ (4) was used instead of nonopsonized gonococci (see below). We also dissected the CL response into extracellular and intracellular events as described by Dahlgren and his colleagues (6, 28). Extracellular CL was measured by adding 1 mM sodium azide, which by itself totally inhibits CL, and 4 U of HRP, an azide-insensitive peroxidase that regenerates an extracellular CL response in the presence of H_2O_2. Intracellular CL was measured by adding 2,000 U of catalase, a high-molecular-weight H_2O_2 scavenger that abrogates extracellular CL by removing H_2O_2. CL was monitored in an LKB-Wallac 1251 Luminometer with the LKB-Wallac 1251/S00-15 Phagocytosis Program.

Measurement of oxygen consumption. Oxygen consumption was measured with Clark oxygen electrodes and a Yellow Springs Instrument oxygen monitor (5) attached to a Perkin-Elmer R100 recorder. Reaction mixtures of 3.5×10^6 to 4.5×10^6 neutrophils in a total volume of 1.5 ml of PBSG were allowed to equilibrate in the 37°C water-jacketed reaction chamber for 10 min before addition of a prewarmed stimulus. A linear rate of oxygen consumption was used to calculate nanomoles of oxygen consumed. Results are expressed as nanomoles of oxygen consumed per 2×10^6 to 3×10^6 neutrophils per minute.

Measurement of HMPS activity. Hexose monophosphate shunt (HMPS) activity was measured by following the oxidation of [1-^{14}C]glucose to $^{14}CO_2$ as described previously (5). [1-^{14}C]glucose was diluted with 50 mM cold glucose to a final specific concentration of 25 μCi/ml (specific activity, 2.8 μCi/μmol) and kept frozen at −20°C until needed. For assays, a 50-fold dilution in 50 mM cold glucose was made just prior to use. To correct for mitochondrial oxidation, [6-^{14}C]glucose oxidation was monitored in the same manner. Reaction mixtures (in 25-ml Erlenmeyer flasks) contained 2×10^6 to 3×10^6 neutrophils, 20 μl of either [1-^{14}C]- or [6-^{14}C]glucose, and PMA (100 ng), OZ (50 to 100 μg), or gonococci in a final volume of 1 ml of PBSG. In all experiments, neutrophils were added last and the reaction flasks were immediately covered with a rubber serum cap (through which a well containing a filter-paper wick and 200 μl of 10% KOH had been inserted). Cell suspensions were shaken at 250 rpm for 30 min in a 37°C water bath. Reactions were stopped, and $^{14}CO_2$ was liberated from buffer by injecting 1 ml of 5 N H_2SO_4 through the rubber cap into the reaction mixture. Flasks were then incubated for an additional 30 min at 37°C with shaking. The rubber caps and wells were then removed, and the wells were placed into 7-ml polyethylene scintillation vials containing 5 ml of Liquiscint (National Diagnostics, Somerville, N.J.). HMPS activity is expressed in terms of counts per minute and represents $^{14}CO_2$ released per 2×10^6 to 3×10^6 neutrophils per 30 minutes. [1-^{14}C]- and [6-^{14}C]glucose were purchased from Amersham Corp., Arlington Heights, Ill.

Reagents. All reagents were purchased from Sigma Chemical Co. unless indicated otherwise.

RESULTS

Stimulation of neutrophil superoxide release by gonococci. To begin characterizing the neutrophil oxidative response to Opa$^+$ gonococci, we measured their ability to induce O_2^- release from neutrophils. Neither Opa4 nor Opa$^-$ gonococci induced O_2^- release at a ratio of 100:1 (gonococci/neutrophil), while 10 ng of PMA per ml induced a high level of O_2^- release (Fig. 1A). Neutrophils pretreated with Opa$^+$ or Opa$^-$ gonococci for up to 100 min could still be stimulated by PMA

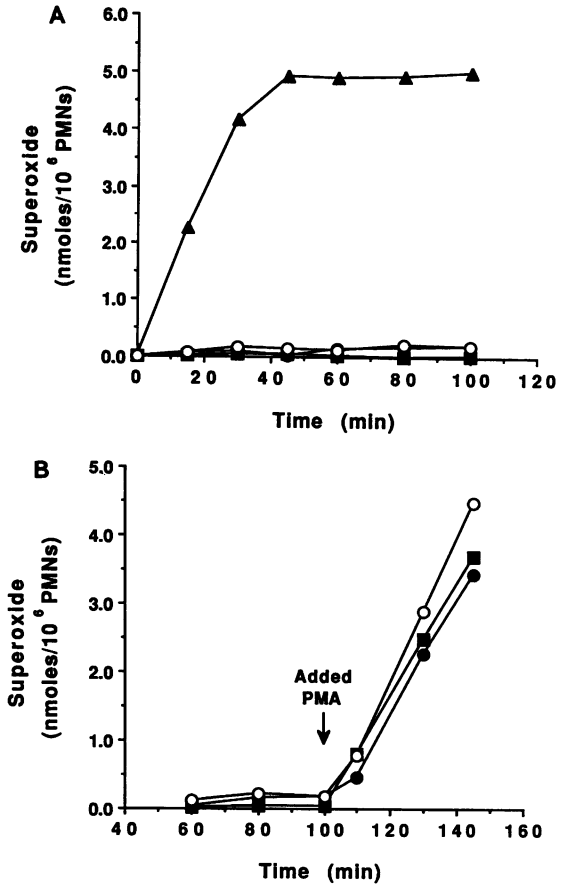

FIG. 1. Gonococci do not stimulate neutrophils to secrete O_2^-. (A) Neutrophils (3×10^6) were incubated with no stimulus (■) or with Opa4 gonococci (1.25×10^8) (●), Opa$^-$ gonococci (1.25×10^8) (○), or 10 ng of PMA (▲) in 1 ml of PBSG, and cytochrome *c* reduction was measured as described in Materials and Methods. (B) Neutrophils (3×10^6) were incubated with no stimulus (■) or with Opa4 (1.25×10^8) (●) or Opa$^-$ (1.25×10^8) (○) gonococci; at the indicated time (arrow), 10 ng of PMA was added to all samples. This is a group of representative experiments performed on at least four separate occasions with almost identical results.

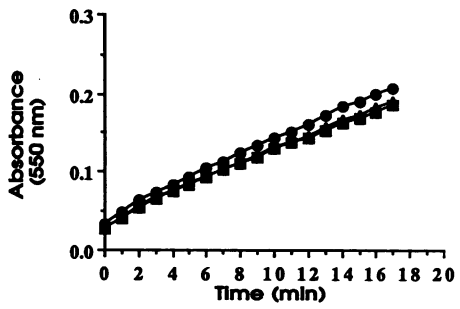

FIG. 2. Gonococci do not scavenge O_2^-. Opa4 gonococci (1.25×10^7 [▲] or 1.25×10^8 [●]) were or were not (■) added to 0.67 μmol of xanthine and 1 mg of cytochrome *c* in 1 ml of PBSG with or without 10 μg of SOD. Xanthine oxidase (0.001 U) was then added to all samples, and the A_{550} was continuously monitored to follow the reduction of cytochrome *c* by O_2^-. Results are expressed as [(A_{550} without SOD) − (A_{550} with SOD)]. This is a group of representative experiments performed on at least four separate occasions with almost identical results.

to release control levels of O_2^- (Fig. 1B). This suggested that gonococci did not inhibit, suppress, or inactivate the oxidative response by neutrophils. Similar results were obtained with formylmethionylleucylphenylalanine or OZ, soluble and particulate activators of neutrophil oxidative metabolism, respectively (data not shown). These data suggest that either Opa$^+$ gonococci do not induce an oxidative burst by neutrophils or there is no release of O_2^- into the surrounding milieu.

To investigate the lack of measurable gonococcus-induced neutrophil O_2^- release further, we examined whether gonococci acted as scavengers of O_2^-. Superoxide was generated (in the absence of neutrophils) by using 0.67 mM xanthine–0.001 U of xanthine oxidase, which yielded a linear rate of cytochrome *c* reduction for ≥20 min. Gonococci did not interfere with the reduction of cytochrome *c* by O_2^- generated by xanthine and xanthine oxidase (Fig. 2). Thus, Opa4 gonococci, at the concentrations used in experiments to investigate neutrophil O_2^- production, do not act as scavengers of O_2^-.

It also occurred to us that the gonococci might be reoxidizing reduced cytochrome *c*, thus making it seem as if no O_2^- was being secreted by stimulated neutrophils. To test this, we reduced cytochrome *c* to about 80% of its A_{550} with supernatants from PMA-stimulated neutrophils. Gonococci (1×10^6 to 2×10^8/ml) were then added to the prereduced cytochrome *c*, and at various times aliquots were withdrawn, the gonococci were removed by centrifugation (6,000 × *g*, 1 min, room temperature), and the A_{550} was recorded. Gonococci did not oxidize prereduced cytochrome *c* (Fig. 3). Hydrogen peroxide was added to some samples to reoxidize prereduced cytochrome *c* to demonstrate that cytochrome *c* was still chemically active (Fig. 3). In all cases, cytochrome *c* was reoxidized to the starting (oxidized) absorbance. Similar results were obtained when cytochrome *c* was prereduced chemically by sodium borohydride instead of neutrophil supernatants (data not shown).

These data suggest that gonococci (i) do not induce O_2^- release from neutrophils, (ii) do not impair the ability of

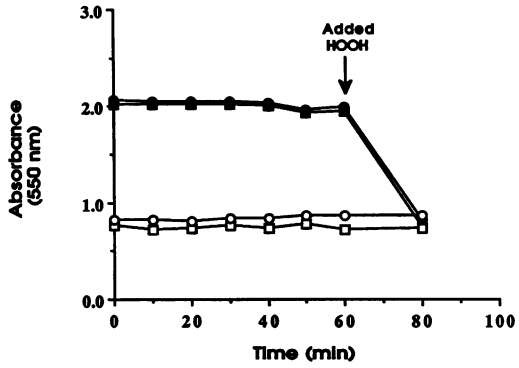

FIG. 3. Gonococci do not oxidize prereduced cytochrome *c*. Cytochrome *c* (1 mg/ml in PBSG) was prereduced to 80% of its extinction coefficient as described in Materials and Methods. Subsequently, 0 (■) or 1.25×10^8 (●) Opa4 gonococci were added and the A_{550} was continuously monitored. At 60 min, H_2O_2 was added to all samples to a final concentration of 1 mM. Samples containing oxidized cytochrome *c* and gonococci (○) and prereduced cytochrome *c* plus 1 mM H_2O_2 (□) were added for reference. Results represent the mean A_{550} ± standard deviation (n = 4). The symbol sizes are larger than the error bars.

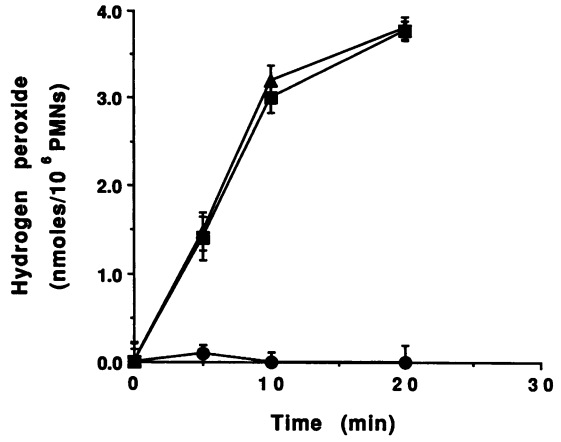

FIG. 4. Gonococci do not stimulate neutrophils to secrete H_2O_2. Neutrophils (3×10^6/ml), 4 μM scopoletin, and 22 nM HRP in 1.5 ml of PBSG were incubated for 5 min at ambient temperature before the addition of either 100 ng of PMA (▲), 1.25×10^8 Opa4 gonococci (●), or both (■). The decrease in relative fluorescence of scopoletin was monitored as described in Materials and Methods. Results are expressed ± standard deviation ($n = 4$).

neutrophils to respond oxidatively to subsequent stimulation by a variety of stimuli, and (iii) do not interfere with the reduction or oxidation of cytochrome *c* by O_2^-.

Stimulation of neutrophil hydrogen peroxide release by gonococci. It is possible that we were not able to measure O_2^- release from neutrophils exposed to Opa^+ gonococci because (i) gonococci were producing SOD, even though there is strong evidence suggesting they do not (2); (ii) O_2^- was spontaneously dismutating to H_2O_2 (3); or (iii) H_2O_2 was being produced in the absence of O_2^- production. Therefore, we measured the release of H_2O_2 from neutrophils stimulated with Opa^+ gonococci. Gonococci did not induce neutrophils to release H_2O_2 into the surrounding milieu (Fig. 4). As with O_2^- release, gonococci did not impair the ability of neutrophils to respond to stimulation by PMA, as demonstrated by the release of similar amounts of H_2O_2 in the presence or absence of gonococci (Fig. 4).

Stimulation of neutrophil LDCL by gonococci. Classically, neutrophil LDCL has been used to directly measure neutrophil oxidative metabolism induced by the gonococcus and by other bacteria (15, 18, 28). To investigate why we were seeing substantial LDCL, but no O_2^- or H_2O_2 release induced by Opa^+ gonococci, we measured intracellular versus extracellular LDCL (6, 28). Extracellular CL was measured by adding 1 mM sodium azide (which by itself totally inhibited LDCL [data not shown]) and 4 U of HRP, an azide-insensitive peroxidase that regenerates an extracellular LDCL response in the presence of H_2O_2. Intracellular CL was measured by adding 2,000 U of catalase per ml, a high-molecular-weight H_2O_2 scavenger that abrogates extracellular LDCL by removing H_2O_2. In the presence of sodium azide and HRP, LDCL was inhibited >80% compared with controls (Table 1). On the other hand, in the presence of catalase alone, LDCL was inhibited <20% compared with controls (Table 1). We conclude that the majority of gonococcus-induced LDCL occurs intracellularly as a result of phagocytosis followed by phagosome-lysosome fusion and the localized production and intravacuolar release of reactive oxygen intermediates (ROI). These data also suggest that ROI are produced as a result of this gonococcus-neutrophil interaction but are not released into the surrounding milieu.

TABLE 1. Intracellular and extracellular LDCL induced by Opa4 gonococci[a]

Sample	Peak CL (mV)		
	No additive	HRP + azide (% of control)	Catalase (% of control)
PMNs alone	4.2 ± 0.4	5.7 ± 2.1 (136)	3.6 ± 0.6 (87)
+ Live GC	153.3 ± 15.9	27.5 ± 2.4 (18)	133.7 ± 4.7 (87)
+ Dead GC[b]	188.6 ± 20.4	37.1 ± 8.4 (20)	161.6 ± 12.3 (88)

[a] Intracellular LDCL was measured in the presence of 2,000 U of catalase per ml, and extracellular LDCL was measured in the presence of 4 U of HRP and 1 μg of azide per ml, as described in Materials and Methods. After addition of gonococci (GC; 10^8/ml) to neutrophils (PMNs; 2×10^6/ml), LDCL was continuously monitored until peak CL responses were recorded. Results are expressed ± standard deviation ($n = 6$). Numbers in parentheses represent percent LDCL of control neutrophils that were not treated with any additive.

[b] Gonococci (4×10^8/ml) were exposed to gentamicin (100 μg/ml) at 37°C for 1 h. This treatment effectively killed >98% of the gonococci as measured by loss of ability to form colonies.

We also monitored the kinetics of LDCL induced by Opa^+ gonococci and compared it with the kinetics of LDCL induced by PMA or OZ. Figure 5 shows the LDCL obtained

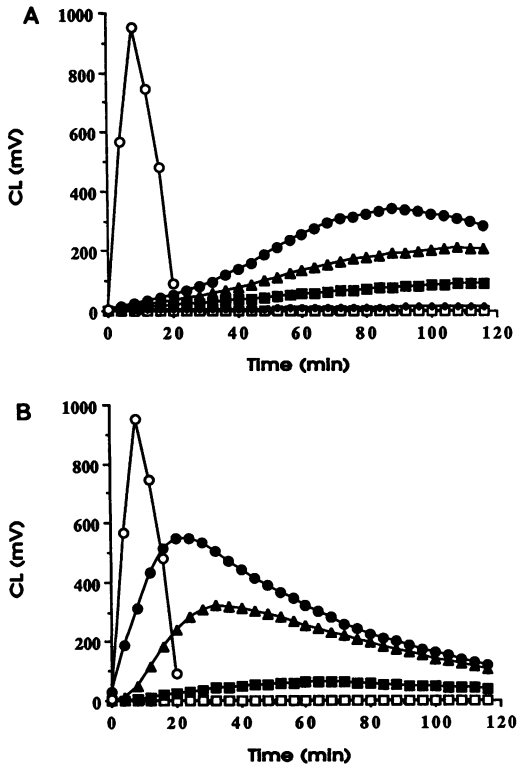

FIG. 5. Kinetics of neutrophil LDCL. (A) Neutrophils (3×10^6/ml) and luminol (10^{-5} M) were incubated without Opa4 gonococci (□) or with Opa4 gonococci at 2×10^6 (◆), 2×10^7 (■), 5×10^7 (▲), or 10^8 (●) per ml or with 10 ng of PMA per ml (○); (B) neutrophils (3×10^6/ml) and luminol (10^{-5} M) were incubated without OZ (□) or with OZ at 5 (■), 50 (▲), or 125 (●) μg/ml or with 10 ng of PMA per ml (○). This is a group of representative experiments done on at least four separate occasions with similar results.

with various concentrations of gonococci (Fig. 5A), various concentrations of OZ (Fig. 5B), or 10 ng of PMA per ml (Fig. 5A and B). It is quite evident that the kinetics of LDCL induced by the three stimuli are very different. It took ≥80 min to reach peak LDCL when neutrophils were stimulated with Opa$^+$ gonococci (10^8/ml), whereas it took ≤10 and ≤25 min to reach peak LDCL when neutrophils were stimulated with PMA (10 ng/ml) or OZ (125 μg/ml), respectively. This suggests that distinct mechanisms are used by each stimulus. Similar results were obtained for all stimuli used when 10^{-5} M lucigenin was used in place of luminol (data not shown).

Stimulation of neutrophil oxygen consumption by gonococci. When gonococci and neutrophils are mixed together, oxygen consumption increases substantially (7); however, it remains unclear to what extent, if any, neutrophil (as opposed to gonococcal) oxygen consumption increases. To identify the source of ROI involved in LDCL, and to quantitate gonococcal versus neutrophil oxygen metabolism, oxygen consumption was directly monitored.

To study this specific problem, we selectively inhibited gonococcal oxygen metabolism and then attempted to stimulate neutrophil oxidative metabolism. In our first approach, we used 100 μg of gentamicin per ml to kill gonococci. Gentamicin decreased gonococcal viability >98%, as determined by the ability of gentamicin-treated gonococci to produce colonies, and decreased gonococcal oxygen consumption by 67% (6.66 to 2.22 nmol of O_2 per min). (Gonococcal oxygen consumption was not totally inhibited, because gentamicin killing does not lead to gonococcal lysis. Thus, residual metabolic processes remain partially intact.) When mixed with neutrophils, dead gonococci induced a substantial increase in neutrophil oxygen consumption (from a resting rate of 1.02 to 11.55 nmol of O_2 per min) compared with live gonococci, which induced an O_2 uptake of 17.05 nmol/min. Although the O_2 consumption observed with live gonococci and neutrophils is surely due to both gonococci and neutrophils, we hypothesize that the increased O_2 consumption seen with dead gonococci is due mostly, if not totally, to stimulated neutrophil oxygen metabolism.

To support the above observations, we used KCN as a probe of oxygen metabolism. KCN (1 mM) totally inhibited gonococcal O_2 uptake, whereas it had a minimal effect on neutrophil O_2 consumption (Table 2). This was expected since stimulated neutrophil O_2 uptake is due mostly to activation of a KCN-insensitive NADPH oxidase, not to mitochondrial (KCN-sensitive) metabolism (4, 7). In the presence of 1 mM KCN, neutrophil O_2 metabolism increased 6.5 times when mixed with live gonococci and 7.5 times when mixed with dead gonococci. These data strongly suggest that Opa$^+$ gonococci stimulate the oxidative burst of human neutrophils. To support these conclusions, we measured the ability of gonococci to induce neutrophil glucose oxidation via the HMPS.

HMPS activity. The HMPS is responsible for supplying NADPH to the NADPH oxidase and is a direct measure of the neutrophil oxidative burst. Gonococcus-induced neutrophil HMPS activity was monitored by following the oxidation of [1-^{14}C]glucose to $^{14}CO_2$ as described in Materials and Methods. [6-^{14}C]glucose oxidation was always monitored parallel to [1-^{14}C]glucose oxidation to indicate changes in glycolysis and the Embden-Meyerhof pathway. During the course of the studies, [6-^{14}C]glucose oxidation did not increase significantly upon activation of neutrophil oxidative metabolism and was always <10% of [1-^{14}C]glucose oxidation (data not shown). Since gonococci, like neutrophils, can also metabolize [1-^{14}C]glucose via the HMPS, we used gentamicin-killed gonococci to monitor the glucose utilized by neutrophils via the HMPS. As mentioned above, gentamicin-killed gonococci were not lysed; thus, they maintained about 10% residual HMPS activity compared with live gonococci (data for live gonococci not shown). Dead Opa$^+$ gonococci stimulated neutrophil HMPS activity in a dose-dependent manner (Fig. 6). These data directly support conclusions drawn from Tables 1 and 2 that Opa$^+$ gonococci stimulate neutrophil oxidative metabolism by stimulating oxygen uptake and HMPS activity.

TABLE 2. Induction of neutrophil O_2 consumption by gonococci[a]

Sample	O_2 consumption (nmol/min/3 × 10^6 PMNs)
PMNs	1.02 ± 0.32
PMNs + KCN	0.92 ± 0.27
Live GC	6.66 ± 2.41
Live GC + KCN	0.00
PMNs + live GC	17.05 ± 2.18
PMNs + live GC + KCN	6.60 ± 1.83
Dead GC[b]	2.21 ± 1.16
Dead GC + KCN	0.0
PMNs + dead GC	11.55 ± 2.36
PMNs + dead GC + KCN	7.92 ± 0.98

[a] Maximal linear rates of O_2 consumption were monitored as described in Materials and Methods. Neutrophils (PMNs; 3 × 10^6/ml) were incubated alone, in the presence of gonococci (GC; 10^8/ml), or in the presence of gonococci plus 1 mM KCN. Also, oxygen consumption of gonococci (10^8/ml) was monitored in the absence or presence of 1 mM KCN. Results are averages ± standard deviations, $n = 4$.

[b] See Table 1, footnote *b*.

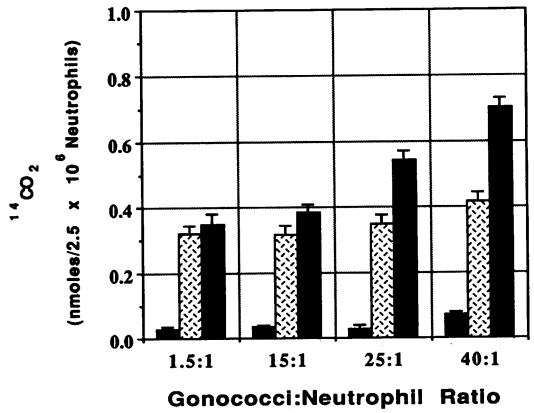

FIG. 6. Gonococci stimulate neutrophil HMPS activity. Neutrophils (2.5 × 10^6) (■), gentamicin-killed Opa4 gonococci ▨, or neutrophils plus gentamicin-killed GC (▨) were incubated in 25-ml reaction flasks, and HMPS activity was measured by following the oxidative decarboxylation of [1-^{14}C]glucose as monitored by the release of $^{14}CO_2$ (described in Materials and Methods). Increasing numbers of gonococci were used so that the final ratios of gonococci/neutrophils were 1.5:1, 15:1, 25:1, and 40:1. Results (30-min assay) represent the mean ± standard deviation ($n \geq 2$). Each mole of glucose that is metabolized through the HMPS stoichiometrically yields 1 mol of CO_2 and 2 mol of NADPH, and each mole of NADPH can reduce 2 mol of O_2 to O_2^-. Therefore, each nmole of $^{14}CO_2$ released correlates to 4 nmol of O_2^- produced.

DISCUSSION

We characterized the oxidative response of neutrophils stimulated by Opa^+ gonococci by investigating some of the molecular events involved in the neutrophil oxidative burst. When neutrophils interact with soluble or particulate matter, including invading bacteria, they respond with a significant increase in oxygen consumption which is commonly referred to as the respiratory burst (4, 12, 13, 18, 47). The ROI produced when neutrophils are activated can be measured by a number of techniques. Many of these techniques involve large detector molecules, and the measurement of oxygen metabolites usually reflects the amount released from the cell (3). In fact, it is generally accepted that the oxidative metabolites produced by the neutrophil plasma membrane-bound NADPH oxidase are released extracellularly (28). However, since the oxygen metabolites are toxic not only to the invading bacteria but also to the surrounding tissue, an effective response to an invading bacterium should be the killing of the bacterium with minimal tissue damage. This means that, for a bacterium-neutrophil interaction to be effective, the ROI produced by the cell should be released inside a phagosome and not into the surrounding milieu.

Initially, we wanted to determine whether neutrophils exposed to Opa^+ gonococci released ROI into the surrounding milieu. Neither Opa4 nor Opa^- gonococci stimulated the release of O_2^- or H_2O_2 from neutrophils nor did they inhibit, suppress, or interfere with the ability of neutrophils stimulated with PMA, formylmethionylleucylphenylalanine, or OZ to release O_2^- or H_2O_2. We conclude from these results that mixing Opa^+ or Opa^- gonococci with neutrophils does not lead to release of ROI into the surrounding milieu. Similar results have been described for the dimorphic fungus *Histoplasma capsulatum*, which fails to trigger release of O_2^- from human neutrophils but causes a respiratory burst (39). These circumstances would not only benefit the host, since many ROI are involved in the inflammatory response, but also the microbe, since it may persist extracellularly in the absence of ROI, which are toxic to many organisms (3). Perhaps both organisms have evolved similar mechanisms for stimulating neutrophils during phagocytosis; however, different neutrophil receptors seem to be involved. *H. capsulatum* apparently binds to the complement receptor CR3 (39), whereas gonococci appear not to use this receptor (16).

Opa proteins are a group of related basic proteins with apparent molecular weights of 24,000 to 30,000 as determined by mobility on polyacrylamide gels. The first Opa (or PII) was described by Swanson as the opacity-associated protein (43). Research from several laboratories, including our own, has shown that most Opa^+ gonococci adhere to human neutrophils and induce a neutrophil CL response, whereas Opa^- gonococci do not (18, 24, 44, 47). We measured the kinetics of LDCL from neutrophils stimulated with Opa^+ gonococci and compared the kinetics with those obtained when neutrophils were stimulated with either PMA, a potent soluble stimulus of LDCL, or OZ, a particulate stimulus of LDCL. The results demonstrated that the kinetics of activation of LDCL for each stimulus was very different. In fact, the kinetics of LDCL induced from Opa^+ gonococci paralleled those of phagocytic killing of Opa^+ gonococci by neutrophils (34); i.e., they were extended over a period of >1 h. We concluded from these studies that gonococcus-induced LDCL could be directly related to phagocytosis of the organism followed by phagosome-lysosome fusion and intracellular localization of ROI.

To investigate this hypothesis, we dissected LDCL into extracellular and intracellular events. This was possible because luminol can diffuse into the neutrophil (9), and the generation of oxygen metabolites resulting from both extra- and intracellular events can be measured (6). Since LDCL is dependent on myeloperoxidase (10, 12), it is possible to exploit this dependence to separate extracellular LDCL from intracellular LDCL. Intracellular LDCL was measured by using catalase, a high-molecular-weight scavenger of H_2O_2 (28) that totally abolishes extracellular LDCL. On the other hand, a combination of azide and HRP was used to measure extracellular LDCL. Azide inhibits the activity of myeloperoxidase, while HRP, being an azide-insensitive peroxidase, restores extracellular LDCL in the presence of H_2O_2 (28). We observed that >80% of LDCL from neutrophils stimulated with Opa^+ gonococci was intracellular. Thus, measurable gonococcus-induced LDCL is temporally related to the events associated with phagocytic killing of the organism. In fact, when similar experiments were performed in the presence of cytochalasin B, which inhibits phagocytosis (11), almost 70% inhibition of LDCL was observed compared with controls (data not shown). This inhibition was not due to a decrease in gonococcal attachment to neutrophils since cytochalasin B does not inhibit adherence of Opa^+ gonococci to neutrophils (15, 16). These data indicate that gonococcus-induced LDCL is a direct result of phagocytosis followed by phagosome-lysosome fusion. The results certainly do not support a hypothesis that gonococci stimulate an oxidative burst simply by adhering to the neutrophil.

Britigan et al. have suggested that the observed increase in oxygen consumption that results from mixing gonococci with neutrophils represents a composite of O_2 consumed by the two cell systems (7). Our data confirm this but, more importantly, we conclusively demonstrate that Opa^+ gonococci stimulate neutrophils to undergo a respiratory burst because (i) neutrophils incubated with live or dead gonococci in the presence or absence of KCN had a higher rate of oxygen consumption compared with neutrophils alone and (ii) neutrophil HMPS activity increased significantly in the presence of dead gonococci. This point is not trivial since many organisms, including *Toxoplasma gondii* (1), *Yersinia pestis* (8), *Brucella abortus* (26), *Salmonella typhi* (25), *Chlamydia trachomatis* (20), and *Mycobacterium leprae* (21), have evolved a survival strategy in which little or no oxidative burst is elicited in the relevant phagocytic cell. We hypothesize that Opa^+ gonococci bind to a neutrophil receptor which is specialized for internalization without induction of superoxide release, similar to that observed for binding of *H. capsulatum* to CR3 (39), as mentioned above. This phenomenon may represent a mechanism by which the gonococci as well as other pathogens survive host defense systems in the extracellular environment and cause persistent infections.

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