

## Interactions of *Neisseria gonorrhoeae* with Human Neutrophils: Effects of Serum and Gonococcal Opacity on Phagocyte Killing and Chemiluminescence

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Serum-sensitive strains of *Neisseria gonorrhoeae* were incubated with suspensions of normal or chronic granulomatous disease human neutrophils in the absence or presence of fresh or heat-inactivated human serum; phagocytosis, gonococcal viability, and chemiluminescence were measured. Nonpiliated opaque or transparent gonococci (colony types 3 and 4, respectively) were used for phagocytic bactericidal assays. In the presence of 2.0% fresh human serum, normal neutrophils killed >90% of types 3 and 4 gonococci by 135 min. Serum alone at this concentration was not bactericidal. In the absence of serum, type 4 gonococci were not killed, whereas type 3 gonococci were killed to the same degree as in the presence of serum. Interestingly, heat-inactivated normal serum slightly inhibited phagocytic killing of type 3 gonococci. Results almost identical to those above were obtained when 5% fresh human serum deficient in complement component 7 was substituted for 2% normal autologous serum. This indicated that the later components of complement were not involved in the observed results. To investigate the mechanisms responsible for the intracellular killing of the gonococci, we used neutrophils from patients with chronic granulomatous disease. These neutrophils are deficient in an activable NADPH oxidase and do not produce bactericidal oxygen products upon phagocytic stimulation. Neutrophils from two unrelated boys with chronic granulomatous disease killed type 3 and 4 gonococci to the same degree as did normal neutrophils. As with normal neutrophils, serum was needed for killing type 4 organisms. As expected, neutrophils from these patients showed absolutely no increased chemiluminescence in the presence of type 3 or 4 gonococci, with or without serum. The effects of serum on gonococcus-induced chemiluminescence by normal neutrophils was also investigated. For these studies, in addition to type 3 and 4 gonococci, we also used transparent colony types of lightly (type 1) and heavily (type 2) piliated organisms. Chemiluminescence induced by type 1, 2, or 3 gonococci (i.e., gonococci possessing either pili or opacity-associated proteins, but not both) was augmented only slightly by serum and then only at low ratios of gonococci to neutrophils. On the other hand, chemiluminescence induced by type 4 gonococci (i.e., gonococci possessing neither pili nor opacity-associated proteins) was substantially increased in the presence of serum. Stimulation of chemiluminescence by type 1, 2, 3, or 4 gonococci was dose dependent in the absence or presence of serum. Heat-killed type 3 gonococci induced chemiluminescence to the same degree as did viable organisms. Since the gonococci used in this research was strongly catalase positive, as are gonococci in general, and since it was killed by chronic granulomatous disease neutrophils, the results indicate that gonococci can be effectively killed within neutrophils, i.e., within phagolysosomes, by nonoxidative bactericidal mechanisms. Whereas type 3 gonococci were phagocytized and killed by neutrophils equally well with or without serum, serum was obligatory for phagocytic killing of type 4 gonococci, i.e., gonococci lacking opacity-associated proteins. In addition, either pili or opacity-associated proteins were apparently necessary for maximal stimulation of neutrophil chemiluminescence. The submaximal stimulation of chemiluminescence by gonococci lacking both pili and opacity-associated proteins, i.e., type 4 gonococci was augmented by low concentrations of nonimmune serum.

Much of the work on phagocytosis and killing of gonococci by human neutrophils has been concerned with antiphagocytic factors of virulent, i.e., piliated, colony types (1-3, 13, 14, 24-26) or with the possible survival of intraleukocytic gonococci (12, 28). James and Swanson (4), King and Swanson (7), and Swanson and colleagues (21-23), and more recently Lambden et al. (9) have suggested that certain gonococcal opacity-associated outer membrane proteins may be more important than pili (or fimbriae) in the association or adherence of the gonococci to various cell types, including human buccal epithelial cells and human leukocytes. Gonococci possessing these specialized outer membrane proteins are readily identified by colony morphology as described by Swanson (22) and Swanson et al. (23). Rosenthal et al. have also observed nonpili-associated components involved in neutrophil-gonococcus interactions (19).

Whereas the effects of pili on phagocytic killing by neutrophils have been intensely studied over the years, the effects of opacity-associated proteins have not. Swanson et al. (23) and King and Swanson (7) studied the effects of opacity-associated proteins on leukocyte association; however, killing was not studied. Gibbs and Roberts (3), Krieger et al. (8), and Schiller et al. (20) characterized the interaction of egg-grown strains from disseminated gonococcal infections with human neutrophils, noting that immunoglobulin G and complement are needed for optimal phagocytosis and killing of type 4 gonococci by monolayers of human neutrophils; type 3 gonococci were not studied. In addition, they showed that heat-labile serum factors are necessary for phagocytosis of nonpiliated transparent organisms, whereas piliated organisms are not interiorized by human neutrophils even in the presence of serum. Densen and Mandell found that serum (10%, complement component 7 [C7] deficient) was required for killing by human neutrophils of type 3 gonococci (strain F62 from an uncomplicated infection) grown for 12 to 14 h in agar and that type 1 gonococci were not killed even in the presence of serum (1). However, Thomas et al. (25) and Thongthai and Sawyer (26) observed significant killing by leukocytes, in the absence of fresh serum, of type 4 gonococci (strain F62) grown for 16 to 18 h on agar, and Swanson and Zeligs (24), using electron microscopy, observed phagocytosis of both piliated and nonpiliated gonococci by human neutrophils in the absence of serum.

Differences also have been found in the effects of serum and gonococcal piliation on the metabolic stimulation of neutrophils by gonococci. Densen and Mandell, using the system described above, found substantial stimulation

of neutrophil hexose monophosphate shunt activity and of O<sub>2</sub> consumption by type 3 gonococci only in the presence of fresh human serum, whereas type 1 gonococci stimulated the oxidative burst in the absence or presence of serum (1). Maximal stimulation by both types was similar. Krieger et al. using an egg-grown disseminated gonococcal infection strain, showed that in the presence of fresh human serum, type 4 gonococci stimulated O<sub>2</sub> consumption 3.5-fold more than did type 2 gonococci (8).

There are situations in which infection occurs in the relative absence of serum immunoglobulin G or complement. Two such well-studied infections are gonococcal urethritis and cervicitis. Concentrations of immunoglobulin G and complement components in urethral or cervical discharges during gonococcal infections vary widely (10, 27). Whatever the estimates, the concentrations are much lower than immunoglobulin and complement concentrations in serum, and it is not known whether these concentrations are effective for optimal opsonization.

The experiments described in this report investigated the effects of serum and gonococcal opacity on phagocytic killing and stimulation of chemiluminescence (CL) by human neutrophils. Data presented below suggest that gonococcal opacity-associated protein(s) actively participate in inducing phagocytic killing and CL by human neutrophils in the absence of serum. Experiments with chronic granulomatous disease (CGD) neutrophils strongly indicate that neutrophils kill gonococci by nonoxidative bactericidal mechanisms.

## MATERIALS AND METHODS

*Neisseria gonorrhoeae*. Gonococci were grown for 16 to 20 h on GC medium base (Difco Laboratories, Detroit, Mich.) with added supplements in a humidified incubator at 36°C in 6% CO<sub>2</sub> in air, as described previously (16). Various colony types were selectively transferred daily on GC medium base according to the criteria of Kellogg et al. (5, 6) and Swanson (21) for piliation and opacity studies. For phagocytic bactericidal studies we used nonpiliated opaque (type 3) and transparent (type 4) colonies. We have previously shown by sodium dodecyl sulfate-polyacrylamide gel electrophoresis that the type 3 gonococci used in this study possess two prominent opacity-associated proteins (of approximately 29,000 and 31,500 daltons) and that these two proteins are absent from type 4 gonococci (17). For CL studies, we also used transparent lightly piliated (type 1) and transparent heavily piliated (type 2) colonies. Opaque type 1 and 2 colonies were not used in CL studies. Strain F62 (serum sensitive) was obtained from P. F. Sparling, University of North Carolina, Chapel Hill. Although not presented, data similar to those described in this report were obtained with a cervical isolate of *N. gonorrhoeae* obtained in 1977 from the clinical laboratories at the University of Arizona Health Sciences Center, Tucson. This strain

has responses to serum or erythromycin that are similar or identical to those of strain F62, and it is used routinely in our laboratory to demonstrate that results are not simply characteristic of strain F62. Data obtained from experiments with this strain gave results that were similar or identical to those obtained with strain F62 (17).

Logarithmic-phase gonococci were obtained by inoculating 20 ml of warm GC broth containing supplements and 400  $\mu\text{g}$  of  $\text{NaHCO}_3$  per ml with 5 ml of a suspension of gonococci grown on plates for 16 to 20 h (optical density at 550 nm, 0.3; Bausch and Lomb Spectronic 20 spectrophotometer; Bausch & Lomb, Inc., Rochester, N.Y.). The mixture was then incubated in a rotary shaker water bath at 36°C for approximately 3 h to a Klett reading (green filter) of approximately 120 ( $4 \times 10^8$  to  $5 \times 10^8$  colony-forming units per ml).

**Neutrophils and serum.** Neutrophils were obtained from apparently healthy adult volunteers and from two unrelated males (13 and 4 years old) with CGD, with informed consent, as approved by the University of Arizona Human Subjects Committee. All normal donors denied previous gonococcal infections. Upon phagocytic stimulation by *Staphylococcus aureus* 502A, the CGD neutrophils showed no increase in oxygen uptake, Nitro Blue Tetrazolium reduction, or CL. Briefly, heparinized whole blood (9 ml) was layered over 5 ml of a Ficoll-Hypaque mixture (containing 6.1 ml of 75% Hypaque and 25.9 ml of 9% [wt/vol] Ficoll in water) and centrifuged at  $200 \times g$  for 30 min at room temperature. Neutrophils were recovered from a cloudy layer just above the large pellet containing erythrocytes and were washed twice at  $200 \times g$  for 7 min at room temperature. Contaminating erythrocytes were not lysed. Cell suspensions contained  $\geq 93\%$  neutrophils and were  $\geq 98\%$  viable as determined by exclusion of 0.25% trypan blue. Purified neutrophils were suspended in medium 199 (pH 7.4) for initial experiments and in Hanks balanced salt solution containing 0.1% gelatin (wt/vol) for later experiments. No differences in phagocytosis or CL results were seen with the two buffers.

Serum was separated from whole blood with integrated serum separation tubes (CORVAC; Monoject, St. Louis, Mo.) according to the directions of the manufacturer and was stored on ice until used. When needed, serum was inactivated by incubation at 56°C for 30 min and then kept on ice until used. All sera used in this study had similar antigenococcal activity, indicating a lack of significant specific antibody.

**Phagocytosis.** Phagocytosis was performed in Falcon snap-capped polypropylene tubes (12 by 75 mm; Becton, Dickinson & Co., Cockeysville Md.) rotating end-over-end at 12 rpm in air at 37°C. Phagocytosis mixtures contained  $5 \times 10^6$  neutrophils,  $5 \times 10^6$  gonococci and, when appropriate, 2.0 to 2.5% (vol/vol) fresh or heat-inactivated autologous serum in 1 ml of medium 199 or Hanks balanced salt solution with 0.1% gelatin. At various times, 10- $\mu\text{l}$  samples were removed, appropriately diluted in GC broth or Hanks balanced salt solution with 0.1% gelatin, and plated on GC medium base plates which were incubated overnight as described above. Colony-forming units were counted at 18 to 24 h.

In some experiments, diluted samples of phagocytosis mixtures were sonicated before being plated to

facilitate the release of intracellular viable gonococci or gonococci attached to the neutrophil membrane. Sonication of diluted samples in plastic tubes (12 by 75 mm) in a cuphorn at power setting 3 for 15 s (model W-225R sonicator; Heat Systems-Ultrasonics, Inc., Plainview, N.Y.) lysed  $\geq 95\%$  of the neutrophils and dispersed clumped gonococci but did not affect gonococcal viability.

**CL.** The method of Rosen and Klebanoff was used to measure CL (18). Linear polyethylene scintillation vials (NEN-938; New England Nuclear Corp., Boston, Mass.) contained 1.0 ml of heat-inactivated (56°C, 30 min) fetal calf serum saturated with luminol (ca. 0.2 mg/ml) and the appropriate additions ( $10^6$  neutrophils, gonococci, or serum) in a total of 5 ml of RPMI 1640 (pH 7.4). Vials were counted in a scintillation counter (model LS-230; Beckman Instruments, Inc., Fullerton, Calif.) in the in-coincidence mode, with tritium preset windows. Sample vials were dark adapted for 30 min, and reactions were initiated by the addition of gonococci (and serum when appropriate). Each vial was counted for 0.2 min, and the counting cycle was repeated at room temperature without further mixing.

## RESULTS

**Phagocytosis.** During preliminary investigations of phagocytosis and killing, we attempted to qualitatively differentiate internalized gonococci from attached gonococci. Smears of phagocytosis mixtures (0.20 ml, containing  $10^6$  neutrophils) were made with a Cytospin cytocentrifuge (Shandon Southern Products, Ltd., Runcorn, England) and stained with Wright stain. Microscopic examination revealed that phagocytized gonococci appeared to be within large, "loose" phagosomes, whereas apparently nonphagocytized but attached gonococci had no visible membrane or space surrounding them. Cytocentrifuge preparations of phagocytosis mixtures that contained  $5 \times 10^5$  neutrophils and  $5 \times 10^6$  type 3 gonococci and were stopped at 0 time and preparations of phagocytosis mixtures that contained 5  $\mu\text{g}$  of cytochalasin B per ml or were incubated on ice for 60 min showed less than 5% of the intraphagosomal gonococci found in 60-min controls. These qualitative observations, in conjunction with results presented below, led us to believe that the killing of gonococci by neutrophils in this system was indeed a consequence of phagocytosis and not of extracellular events.

**Phagocytic killing.** Type 3 gonococci were killed over time by suspensions of human neutrophils in the presence or absence of serum (Fig. 1), whereas type 4 gonococci were killed only in the presence of serum. These results were observed with logarithmic-phase broth-grown bacteria or with plate-grown cells harvested at 16 to 20 h. Initially, phagocytosis mixtures were sonicated after dilution and before plating (as described above) to release intracellular gonococci and to disperse any clumps

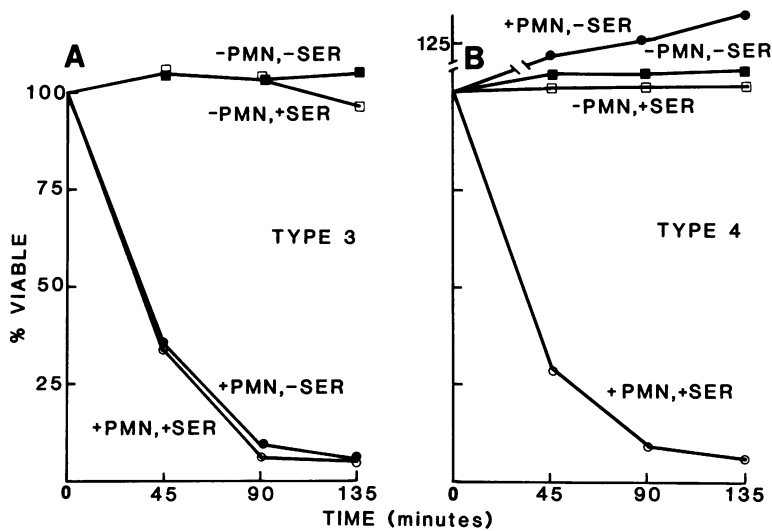


FIG. 1. Viability of type 3 (A) and type 4 (B) gonococci in the presence (+) or absence (-) of purified human neutrophils (PMN), with (+) or without (-) 2% fresh autologous serum (SER).

that might have formed during the incubations. Sonication of samples did not alter the recovery of viable gonococci from phagocytic bactericidal assays. Therefore, sonication was not used in most of the assays described in this report. Since type 3 gonococci were killed as well by human neutrophils in the absence of serum as in the presence of serum, we were interested in knowing whether our purified human neutrophils were somehow being activated by our purification methods, perhaps by binding with serum constituents such as activated complement components. Neutrophils purified by Ficoll-Hypaque sedimentation or by Percoll sedimentation yielded identical results when used in phagocytic bactericidal assays. In addition, prewarming neutrophils at 37°C for 30 min and then washing them twice in Hanks balanced salt solution with 0.1% gelatin did not affect their ability to kill type 3 gonococci in the absence of serum. The maximal concentration of fresh autologous serum that could be used without causing gonococcal death over the 135-min incubation period was 2.0 to 2.5%. The presence of 2.5% heat-inactivated serum partially inhibited the killing of type 3 gonococci. Preincubation of human neutrophils with 5  $\mu$ g of cytochalasin B per ml for 30 min completely inhibited their ability to kill gonococci over the 135-min incubation period. In one set of phagocytosis experiments with type 3 and 4 gonococci, 5% C7-deficient serum was used instead of 2% normal serum to see if an increase in opsonin (C3 and C5) concentration would increase killing; it did not.

**CL.** Since serum appeared to have little effect on phagocytosis and killing of type 3 gonococci

by neutrophils, we were interested in its effects on CL induced by various types of gonococci. Results paralleled those of the phagocytic bactericidal assays in that fresh human serum (2.0 to 2.5%) had little or no effect on CL induced by type 1, 2, or 3 gonococci, except at very low ratios of gonococci to neutrophils (Fig. 2 and 3). Data for the induction of CL by type 2 gonococci were identical to those obtained with type 1 gonococci and are therefore not shown. Increasing numbers of type 1, 2, or 3 gonococci induced increased rates of CL, to a maximal stimulation at a ratio of approximately 10:1 to 20:1, regardless of the presence of serum. At a ratio of 0.5:1, which was the lowest ratio tested that yielded consistent results, serum did increase the induction of CL by gonococci. In contrast to types 1, 2, or 3 gonococci, induction of CL by type 4 gonococci was augmented at least 10-fold in the presence of serum at all ratios tested (Fig. 4). Serum, gonococci, or neutrophils alone did not chemiluminesce ( $\leq 50$  cpm) over the 3-h experimental period. Neutrophils in the presence of serum did occasionally show an increased basal level of CL ( $\leq 5,000$  cpm over 3 h) over that observed for serum or neutrophils alone. Heat-killed (60°C, 60 min) type 3 gonococci stimulated CL to the same degree as did live gonococci, in the absence or presence of serum (data not shown). As was seen with phagocytic killing, the presence of heat-inactivated serum slightly decreased the CL response of neutrophils toward type 3 gonococci.

We also investigated the effects on CL of the 20% heat-inactivated fetal calf serum present in our system. In two experiments, luminol was

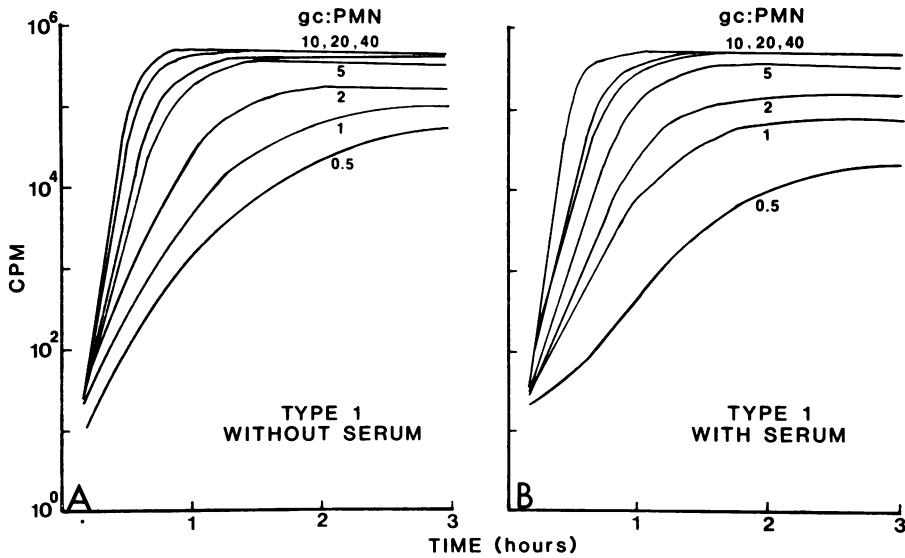


FIG. 2. CL (presented as counts per minute [CPM]) by purified human neutrophils (PMN) induced by type 1 gonococci, in the absence (A) or presence (B) of 2% fresh autologous serum. Various ratios of gonococci (gc) to PMN were used, as indicated by the small numbers above and below the curves.

dissolved to a concentration of  $10^{-2}$  M in dimethyl sulfoxide instead of in fetal calf serum and was added to the CL reaction mixtures at a final concentration of  $10^{-4}$  M. The CL observed in this system with type 3 or 4 gonococci in the absence or presence of 2% fresh autologous human serum was identical to that reported above for the system containing fetal calf serum.

**Phagocytic killing and CL by CGD neutrophils.** To investigate the contribution of nonoxidative

and oxidative bactericidal mechanisms to the intracellular killing of gonococci, we performed phagocytosis experiments with neutrophils obtained from two unrelated male children with CGD. In the presence or absence of serum, killing of type 3 (Fig. 5) or type 4 (data not shown) gonococci was almost identical for both normal and CGD neutrophils; i.e., serum was not needed for killing of type 3 gonococci, whereas it was needed for killing of type 4

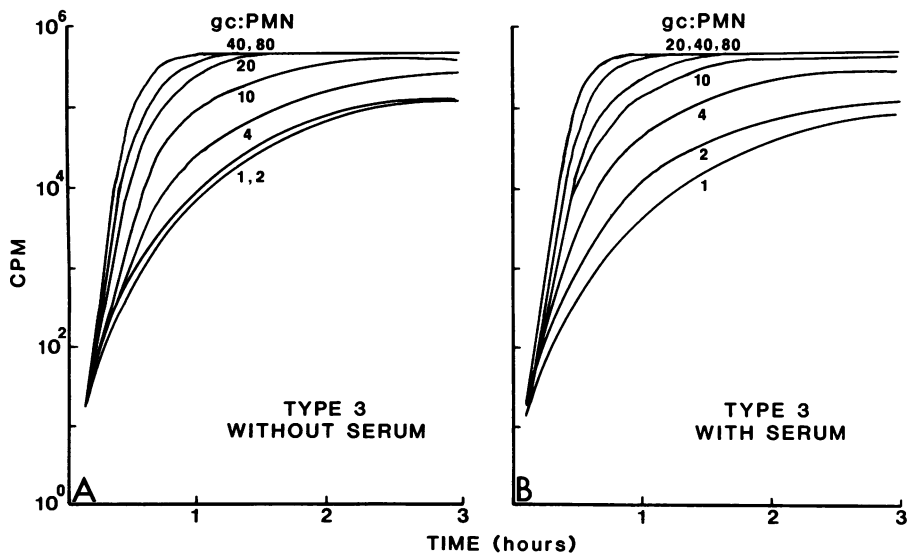


FIG. 3. CL induced by type 3 gonococci. See legend to Fig. 2 for details.

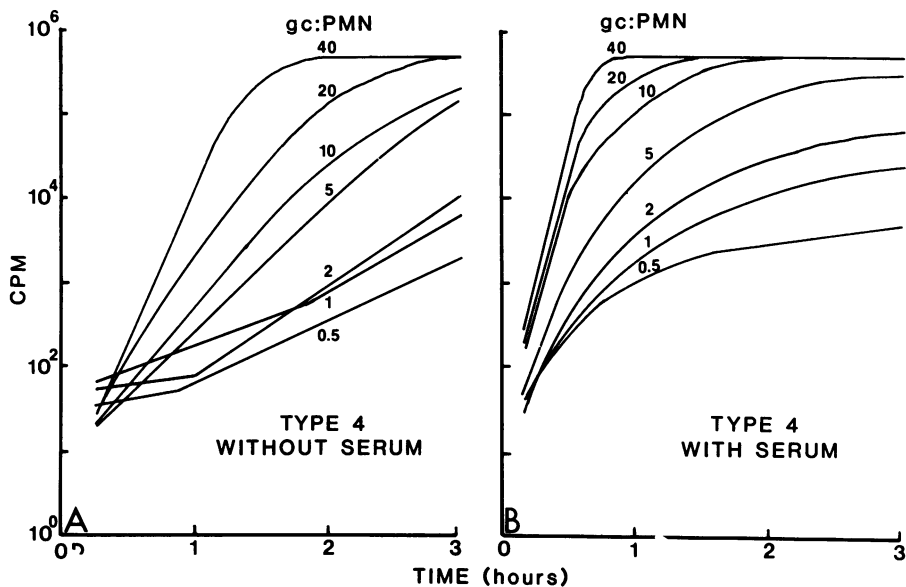


FIG. 4. CL induced by type 4 gonococci. See legend to Fig. 2 for details.

gonococci. In experiments run on the same days as those described above, CGD neutrophils failed to kill *S. aureus* 502A.

Type 3 or 4 gonococci induced no CL ( $\leq 100$  cpm) by CGD neutrophils in the absence or presence of serum at a ratio of gonococci to neutrophils of 10:1 over a 3-h period. In experi-

ments run at the same time, gonococci did induce the expected CL response in control vials containing normal neutrophils.

## DISCUSSION

The data presented in this manuscript support the idea first proposed by Swanson (23) that gonococci possess surface characteristics, be they physical or physicochemical, that favor gonococcal interaction with human neutrophils in the absence of serum. We have previously confirmed that the type 3 gonococci used in this report indeed possess some of the opacity-associated proteins and that type 4 organisms do not contain these proteins (17). The observation that phagocytized gonococci appear to be within loosely-fitting phagolysosomes has been reported by others (1, 3, 8) and, although remaining to be proven, appears to be a rather reliable characteristic in qualitatively determining extracellular versus intracellular gonococci.

Densen and Mandell found that type 3 gonococci were not phagocytized or killed by human neutrophils in the absence of serum (1). This discrepancy is odd since these authors used phagocytosis methods (tumbling tube) similar to ours, agar-grown strain F62 gonococci (the same strain that we used), and growth conditions similar to those used in experiments reported in this manuscript. The gonococci used by these authors were harvested at 12 to 14 h, whereas we harvested agar-grown gonococci at 16 to 20 h. In addition, in the presence of 10% C7-deficient serum, Densen and Mandell observed

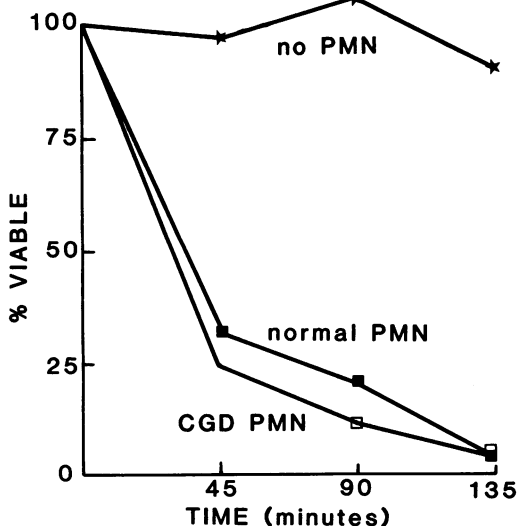


FIG. 5. Phagocytic killing (expressed as percent viability) of type 3 gonococci in the presence of 2% fresh serum by human neutrophils (PMN) isolated from normal and CGD donors.

≥99% killing of gonococci in 1 h, whereas we observed no more than 90 to 95% killing after 2 h, with or without serum. On the other hand, Dilworth et al. observed similar degrees of ingestion (52 to 56%) of type 3 gonococci (strain F62) by monolayers of human neutrophils in the presence of no serum, normal serum, or heat-inactivated serum (2). Killing was not measured.

It is of interest that certain strains of *Peptostreptococcus* are also readily phagocytized by human neutrophils in the absence of serum and that, similar to our observations, heat-inactivated serum actually inhibits phagocytosis (P. Quie and L. Switalski, personal communication.)

Our studies measuring the stimulation of neutrophil CL by gonococci generally supported our phagocytosis and bactericidal results. With types 1, 2, or 3 gonococci, i.e., gonococci possessing either pili or opacity-associated proteins, at gonococcus-to neutrophil ratios of >4:1, CL was not augmented by the presence of serum. On the other hand, CL induced by type 4 gonococci was stimulated by the addition of serum at all ratios tested by at least 10-fold and by as much as 500-fold. Densen and Mandell (1) and Krieger et al. (8) have reported on the effects of serum on the ability of gonococci to induce O<sub>2</sub> consumption and hexose monophosphate shunt activity (measured by glucose oxidation) in human neutrophils. Unfortunately, conclusions reached by the two groups of investigators are dissimilar and inconsistent with the CL results obtained in the present studies. Krieger et al., using egg-grown disseminated gonococcal infection strains, found that, in the presence of 10% serum, logarithmic-phase type 4 gonococci cause a greater increase in metabolic activity than do type 2 gonococci; they did not use type 3 gonococci in their studies (8). Densen and Mandell, using agar-grown, serum-sensitive strains, observed few differences between type 1 and type 3 gonococcal stimulation of oxidative metabolism in the presence of serum; in the absence of serum they found that type 1 gonococci were far superior to type 3 gonococci in stimulating the oxidative burst (1).

Our data suggest that serum does not affect the ability of piliated transparent or nonpiliated opaque gonococci to stimulate CL, except at very low ratios of gonococci to neutrophils. The biological relevance of the minimal CL observed at these low ratios can be debated. One must keep in mind that the results in Fig. 2, 3, and 4 are presented on a logarithmic scale. Stimulation by serum of CL induced by type 1, 2, or 3 gonococci occurred only when neutrophils were producing 0.3% of the maximal experimental CL measured.

The interactions of gonococci with CGD neutrophils were unexpected. Since gonococci are

strongly catalase positive (11), it would be hypothesized that they would not be killed within CGD neutrophils, since there would be no available H<sub>2</sub>O<sub>2</sub> (15). On the contrary, gonococci were killed as well by CGD neutrophils as they were by normal neutrophils. The explanation for this is presently unknown; however, the results strongly suggest that nonoxidative mechanisms play an important role in the intraleukocytic killing of *N. gonorrhoeae*. It has been shown previously that type 1 and 4 gonococci are readily killed in vitro by human neutrophil granule contents, by apparently nonoxidative mechanisms (16). It has also been shown that *Pseudomonas aeruginosa*, another gram-negative, catalase-positive organism, is killed equally well by normal neutrophils, CGD neutrophils, and normal neutrophils under anaerobic conditions (B.M. Holmes-Gray, RES J. Reticuloendothel. Soc. 22:87-88, 1977).

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