Gonococcal Interactions with Polymorphonuclear Neutrophils

IMPORTANCE OF THE PHAGOSOME FOR BACTERICIDAL ACTIVITY

PETER DENSEN and GERALD L. MANDELL, *Department of Medicine, University of Virginia School of Medicine, Charlottesville, Virginia 22901*

ABSTRACT Gonococci are capable of attaching to the surface of polymorphonuclear leukocytes (PMN). In this location they resist phagocytosis and are not killed by PMN. To delineate the factors involved in the survival of these gonococci, we investigated the interaction of virulent gonococci, which adhere to cells and resist phagocytosis, and avirulent gonococci, which are phagocytized and killed by PMN.

In the presence of serum, both virulent and avirulent gonococci associate equally well with PMN and stimulate increases in oxidative metabolism. In the absence of serum virulent gonococci attached to PMN and stimulated PMN oxidative metabolism to a greater extent than avirulent gonococci which did not attach to PMN ($P = 0.0009$). Therefore, the survival of virulent gonococci attached to the PMN surface is not a result of failure to activate oxidative and bactericidal mechanisms.

Both virulent and avirulent gonococci stimulated equivalent PMN specific granule release as measured by the appearance of lactoferrin in the media. Phagocytosis of avirulent gonococci stimulated significantly greater β-glucuronidase release ($P = 0.01$) and myeloperoxidase-mediated iodination of protein ($P = 0.001$) by PMN than attachment of virulent gonococci. In the absence of serum neither type of gonococci stimulated β-glucuronidase release or protein iodination by PMN. Thus, virulent gonococci fail to stimulate primary granule release by PMN.

To further assess the role of attachment versus ingestion on the survival of gonococci, PMN were treated with cytochalasin B to block ingestion. Cytochalasin B-treated PMN were unable to kill either virulent or avirulent gonococci despite normal degranulation stimulated by the latter.

The failure of PMN to kill surface-attached gonococci appears to be a consequence of the failure of PMN to enclose the virulent gonococci within a phagosome. The phagocytic vacuole thus plays a critical role in normal PMN bactericidal activity by providing a closed space in which the proper concentration of substances may be achieved to generate microbicidal activity.

This work was presented in part at the National Meeting of the American Federation of Clinical Research at Atlantic City, N. J. in May 1976 and published in abstract form. 1976. *Clin. Res.* **24**: 342A. 1977. *Clin. Res.* **25**: 375A.

Received for publication 8 April 1977 and in revised form 31 July 1978.

INTRODUCTION

Phagocytosis of most bacteria by normal human polymorphonuclear neutrophils (PMN)[1] usually results in the death of the microorganisms. The generation of oxidative metabolites and the release of granule contents from both the specific and the primary granules into the phagosome during phagocytosis is responsible for the killing of ingested organisms by PMN (1). The same substances are also released extracellularly by PMN during phagocytosis and during interaction with a nonphagocytosable stimulus (2–5). Extracellular release of these substances has recently been implicated in the in vitro production of damage and probably death of *Candida albicans* pseudohyphae by PMN (6), in the killing of tumor cells by PMN (7), and has been postulated to play a role in certain types of immune-mediated tissue injury (8). Thus PMN appear capable of exerting a "cidal" effect on intracellular and certain extracellular organisms.

Although it has been classically taught that the gonococci seen in association with PMN in Gram-stained smears of urethral exudate are "intracellular," several studies have demonstrated that many of these organisms are in fact attached to the surface of these cells (9–13). In this extracellular but attached location the

[1] *Abbreviations used in this paper*: CB, cytochalasin B; HBSS, Hanks' balanced salt solution; HMP, hexose monophosphate shunt; PMN, polymorphonuclear neutrophil.

organisms remain viable, apparently immune to microbicidal attack by PMN. To delineate the factors responsible for the survival of attached gonococci we investigated the morphologic and metabolic consequences of PMN interaction with virulent, piliated, type I gonococci which adhere to cells but resist phagocytosis, and with avirulent, nonpiliated, type III gonococci which are readily phagocytized (9–15).

METHODS

Bacteria. Gonococcal colony types I and III (strain F-62, kindly supplied by Dr. Douglas Kellogg, Venereal Disease Research Branch, Communicable Disease Center [U. S. Public Health Service] Atlanta, Ga.) were maintained by clonal selection on gonococcal agar base (BBL, Cockeysville, Md.). The bacteria were harvested at 12–14 h, washed and resuspended in Hanks' balanced salt solution (HBSS; Microbiological Associates, Bethesda, Md.). The bacterial concentration was adjusted to 10^9 organisms/ml with a Gilford 2400 spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) at 580 nm to read an absorbancy of 1.100. Except where noted, the gonococcus to PMN ratio used was 40:1 or greater. Petroff-Hauser chamber counts were performed as an additional method of bacterial quantitation. DNA quantitation by the method of Burton (16) confirmed that both of these methods of adjusting bacterial concentrations yielded equal numbers of type I and type III gonococci.

Neutrophils. Neutrophils were obtained from normal volunteers denying a history of gonorrhea. The PMN were purified by centrifugation over Ficoll-Hypaque (Ficoll, Pharmacia Fine Chemicals, Piscataway, N. J.; Hypaque, Winthrop Laboratories, Sterling Drug Co., New York) (17) and(or) by sedimentation of whole blood in an equal volume of heparinized (10 U/ml) 6% dextran-70 (Pharmacia Fine Chemicals) for 1 h at 22°C at a 60° angle. The leukocyte-rich supernate was removed and centrifuged at 200 *g* for 12 min at 22°C. In the oxygen consumption and degranulation experiments the erythrocytes were then lysed twice with 3 ml of iced 0.22% saline plus heparin (10 U/ml). Isotonicity was restored after 45 s with 0.88 ml of 3% saline followed by 5 ml of HBSS. Centrifugation was repeated and the pellet resuspended in HBSS. The final suspension contained 1.0×10^7 PMN/ml.

Phagocytosis and bactericidal activity. To eliminate any contribution of serum to the bactericidal activity, phagocytosis and killing of gonococci by PMN was assessed in the presence or absence of C_7-deficient human serum (kindly provided by Dr. Terry Lee, University of North Carolina, Chapel Hill, N. C.). This serum has no intrinsic bactericidal activity for gonococci (18, 19). PMN (10^6) and gonococci (10^6) in 1 ml of HBSS containing 10% C_7-deficient serum were tumbled at 12 rpm in 12 × 17 mm polypropylene tubes (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) at 37°C for 60 min. Aliquots were removed after 0, 15, 30, and 60 min of incubation and total bacterial counts were performed after hypotonic lysis of PMN. The same experiments were also performed in the presence of cytochalasin B (CB; Sigma Chemical Co., St. Louis, Mo.). The final concentration of CB (0.5% in dimethylsulfoxide) in the incubation mixture was 5 μg/ml. All experiments were performed a minimum of three times.

Phase contrast microscopy. Samples were prepared as described for the phagocytosis experiments except that incubation was carried out on a glass slide under a cover slip on a temperature-controlled stage of a Zeiss photomicroscope (Carl Zeiss, Inc., New York) with phase optics fitted with a 16-mm Bolex movie camera (Bolex International S.A., Switzerland) and Sage stroboscopic flash (Sage Instruments, Div. Orion Research, Inc., Cambridge, Mass.). This permitted observation and photography of the dynamic gonococcal-PMN interaction.

Electron microscopy. PMN (10^6) and gonococci (10^8) were incubated for 30 min with either 10% autologous serum or no serum, as described in the phagocytosis experiments. At the end of 30 min, phagocytosis was stopped by icing the reaction tube. Pellets were prepared by centrifugation at 200 *g* for 10 min at 0°C and fixed for 24 h at 4°C in 2.5% glutuaraldehyde buffered at pH 7.2 with 0.1 M phosphate. They were postfixed in osmic acid in the same buffer at 4°C for 3 h, dehydrated in graded alcohols and propylene oxide and embedded in Epon 812 (Shell Chemical Co., Houston, Tex.). Thin sections were cut with an ultramicrotome, placed on copper grids, stained with uranylacetate and lead citrate (20), and examined and photographed with a Siemens Elmiskop 1A electron microscope (Siemens Corp., Iselin, N. J.).

Uptake of radiolabeled gonococci by PMN. Gonococcal agar base containing 1 ml of ^{14}C-L-aminoacids (10 μCi/ml; New England Nuclear, Boston, Mass.) per 100 ml of agar was used to grow radiolabeled gonococci. Equivalent concentrations of type I and type III gonococci prepared as described above contained equivalent amounts of radioactivity. ^{14}C-labeled gonococci (10^8) and 10^6 PMN in 1 ml HBSS containing 10% normal human serum were tumbled at 12 rpm for 30 min at 37°C. Aliquots (0.1 cm^3) were removed at 0, 5, 10, 20, and 30 min. The aliquots were added to 0.9 cm^3 of ice cold HBSS and centrifuged for 5 min at 200 *g* at 0°C. The pellet was washed twice more in iced HBSS and then counted in a Beckman LS 250 scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.). Percent uptake was calculated by dividing the ^{14}C cpm in the sediment at each time point by the total number of ^{14}C cpm added at zero time and multiplying by 100.

Oxygen consumption. PMN (10^6) and gonococci (10^7) in 3 ml of HBSS were incubated with 10% serum or no serum in the chambers of a model 53 polarographic oxygen monitor (Yellow Springs Instrument Co., Yellow Springs, Ohio). The incubation mixture contained 1 mM KCN which abolished oxygen consumption of live gonococci without interfering with PMN oxygen consumption or the attachment or ingestion of gonococci to PMN. The oxygen consumption of PMN incubated alone or with heat-killed staphylococcus 502A was also measured under each set of experimental conditions. Data were fed into a linear recorder and the oxygen consumption during 10 min of maximal uptake was measured and expressed as μl O_2/h per 10^7 PMN.

Hexose monophosphate shunt measurements. The reaction was carried out in siliconized 25-ml Erlenmeyer flasks containing 10^6 PMN, 10^8 gonococci, and 0.2 ml ^{14}C-L-glucose (2 μCi/ml) (New England Nuclear) in a final volume of 2 ml. The experiments were conducted in 25% normal autologous serum or in the absence of serum. The flasks were stoppered (Kontes Glass Co., Vineland, N. J.) and the stoppers pierced by (*a*) a center well (Kontes Glass Co.) containing a 2 × 0.5-cm strip of Whatman qualitative 11.0 cm filter paper (W & R, Balston Ltd., England) previously saturated with fresh 10% KOH and (*b*) polyethylene tubing (inside diameter = 0.023 × outside diameter = 0.038 inches; Clay Adams, Parsippany, N. J.). The reaction was initiated by adding the gonococci to the flask through the tubing, flushing with air, and knotting the tubing. The flasks were incubated in a shaker water bath at 20 rpm at 37°C for 30 min. The reaction was stopped and $^{14}CO_2$ released by adding 0.6 ml of 1 N HCl through the tubing, reknotting, and incubating for an additional 15 min. The strips of filter paper were then placed in scintillation fluid (40 ml, Liquiflor, New England Nuclear; 660 ml toluene,

300 ml absolute methanol) and counted in a liquid scintillation counter. Experiments were also performed with heat-killed staphylococcus 502A as the phagocytic stimulus.

Degranulation. Tubes containing 0.4 ml PMN mixture ($1-5 \times 10^7$ PMN/ml), 0.1 ml at either 10^9 gonococci, 10^9 heat-killed *Staphylococcus aureus* 502A or 10^9 zymosan A plus 0.1 ml of either normal autologous serum or HBSS were tumbled for 30 min, immediately iced, and then centrifuged at 2,000 *g* for 5 min at 0°C. The effect of CB (5 μg/ml) on PMN degranulation in serum was also measured. Determinations of granule protein and enzyme activity were performed on the supernate. The sediment was resuspended in 0.3 ml, 0.1 M phosphate buffer, pH 8.0 containing 1.0 M NaCl and 0.3% cetyltrimethylammonium bromide (Sigma Chemical Co.) at 22°C for 30 min with frequent vigorous vortexing to disrupt intact granules.

Myeloperoxidase and lactoferrin concentrations were measured by the Mancini radial immunodiffusion technique (21) as described by Leffell and Spitznagel (22). 5-μl samples were placed in the wells and allowed to diffuse overnight at room temperature in a moist chamber. The plates were then stained for 5 min with 4% tannic acid (23) and the precipitin ring diameters measured. (Purified human lactoferrin and myeloperoxidase and rabbit anti-human lactoferrin and anti-human myeloperoxidase were the generous gifts of Dr. John Spitznagel, University of North Carolina.)

β-glucuronidase activity was determined by the method of Fishman et al. (24) using standard reagents (Sigma Chemical Company). This technique is based on the spectrophotometric determination of the generation of phenolphthalein from phenolphthalein-glucuronic acid by the action of β-glucuronidase.

Myeloperoxidase-mediated iodination by granulocytes. The method of Klebanoff (25) as modified by Root et al. (26) was employed. Experiments were performed in 10% autologous normal serum or, in the absence of serum, in a final volume of 1.0 ml HBSS containing 0.2 ml Na ^{125}I (10 μCi/ml) (New England Nuclear) in addition to 10^6 neutrophils and 10^8 gonococci. Experiments were also performed with CB (5 μg/ml). The reaction mixture was tumbled at 12 rpm at 37°C for 30 min. The reaction was stopped by the addition of 2 ml of iced 20% trichloroacetic acid and the specimen centrifuged at 2,000 *g* at 0°C for 5 min. The pellet was washed twice with 4 ml of iced 10% trichloroacetic acid. The final precipitate was resuspended in 1.0 ml of 10% trichloroacetic acid and counted in a gamma counter. PMN incubated alone or with heat-killed staphylococcus 502A served as controls.

Cell-free peroxidase-mediated iodination. With the method Klebanoff (25), iodination of 10^8 gonococci was performed in a final volume of 1 ml HBSS containing 10% human serum. In addition, the reaction mixture contained 80 U lactoperoxidase (Sigma Chemical Co.), 0.1 ml Na ^{125}I (10 μCi/ml), and 0.1 ml 3% hydrogen peroxide (Parke, Davis & Co., Detroit, Mich.). The reaction mixture was tumbled at 12 rpm at 37°C for 30 min and the determination of bacterial iodination determined as described above.

Statistical methods. Student's *t* test was used to calculate statistical significance in all experiments.

RESULTS

Phagocytosis and bactericidal capacity. After incubation with PMN in 10% C_7-deficient serum there is a 2–3 log decrease in the number of viable type III gonococci. Under the same conditions, there was a minimal decrease in the number of viable type I gonococci (Fig. 1A). Neither type I nor type III gonococci was killed

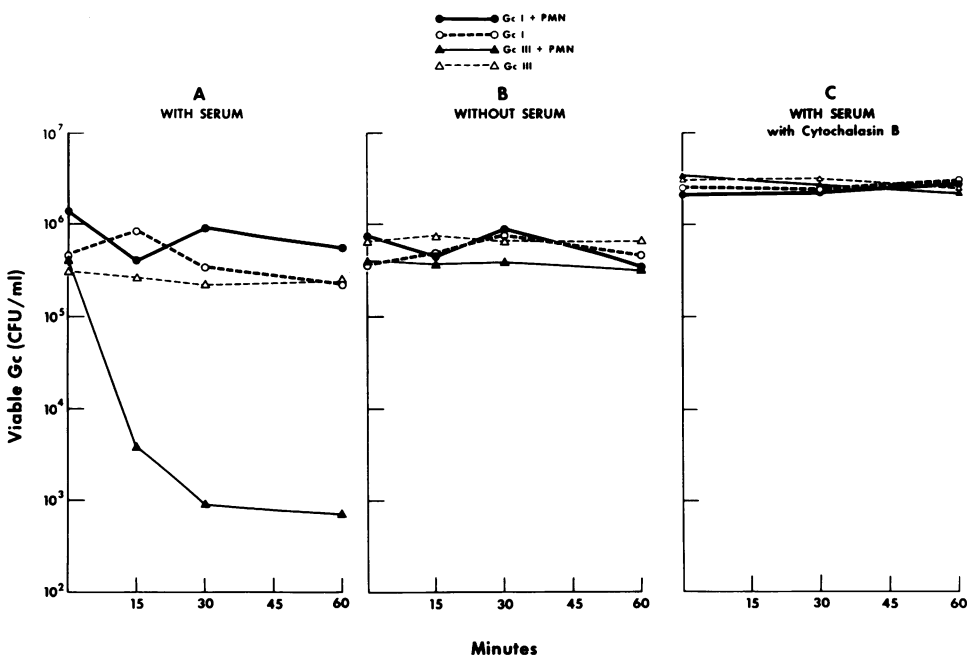

FIGURE 1 Bactericidal activity of human PMN for type I and type III gonococci (GC). In the presence of serum (A) type III gonococci were rapidly killed by PMN, but type I gonococci were not. In the absence of serum (B) and in the presence of serum plus CB (C) there was no killing of either type I or type III gonococci.

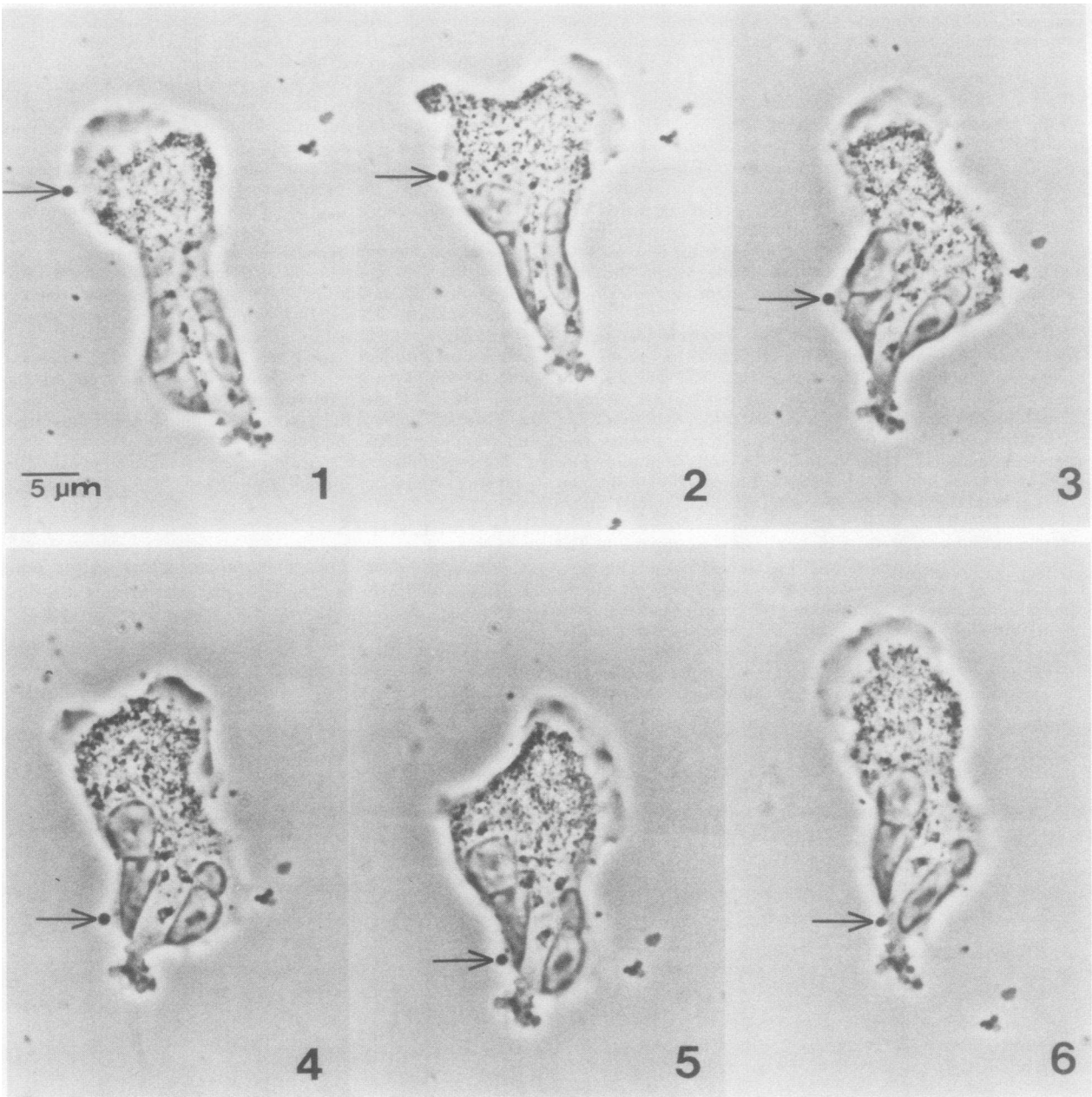

FIGURE 2 Sequential phase contrast micrographs of the PMN-type I gonococcus interaction. Initial contact is made at the granule-free, advancing PMN front. Note the persistent membrane-attached location of the type I gonococcus (arrow) and movement along the membrane to the tail where it remains with other adherent gonococci (total time of 90 s).

in tubes containing serum alone. In the absence of serum (Fig. 1B), there was no killing of either type I or type III gonococci incubated with or without PMN. Neither type of gonococci was killed by CB-treated PMN in the presence of serum (Fig. 1C). More than 95% of the PMN remained viable as determined by trypan blue exclusion.

Phase contrast microscopy. Photomicrographs of the interaction of PMN with type I and type III gonococci in serum are shown in Figs. 2 and 3. Type I gonococci (Fig. 2) adhere to the PMN membrane and move along the membrane to the "tail" of the PMN. In contrast, the type III (Fig. 3) gonocci are ingested by the PMN and can be seen in a phagocytic vacuole.

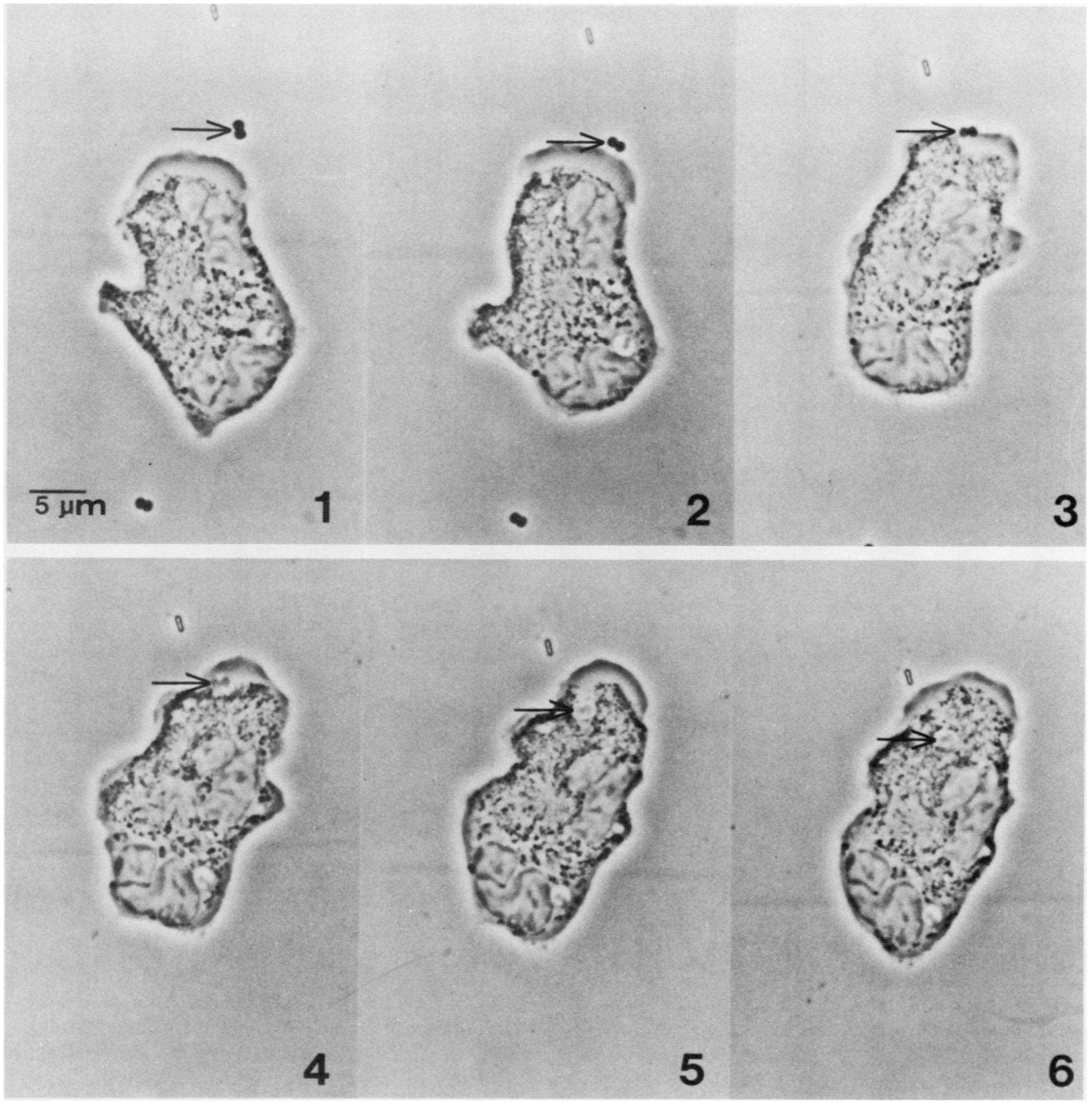

FIGURE 3 Sequential phase contrast micrographs of the PMN-type III gonococcus interaction. Note the ingestion of type III gonococci at the leading PMN edge. The internalized organism is in a phagocytic vacuole and appears to be surrounded by granules (total time of 90 s).

Electron microscopy. The attached but extracellular location of the type I gonococci and the primarily intracellular location of the type III gonococci incubated with serum is demonstrated in these photomicrographs as shown in Figs. 4 and 5, respectively. In Fig. 6 gonococcal pili can be seen in contact with the PMN surface.

Uptake of radiolabeled gonococci by PMN. The uptake of both type I and type III gonococci by PMN (Fig. 7) was equivalent at 10, 20, and 30 min. However, at zero time and at 5 min fewer type I gonococci than type III were cell associated. This early difference in uptake of piliated type I and nonpiliated type III gonococci by PMN is unexplained. All the metabolic and degranulation responses by PMN to gonococci were measured after 30 min incubation, thereby elim-

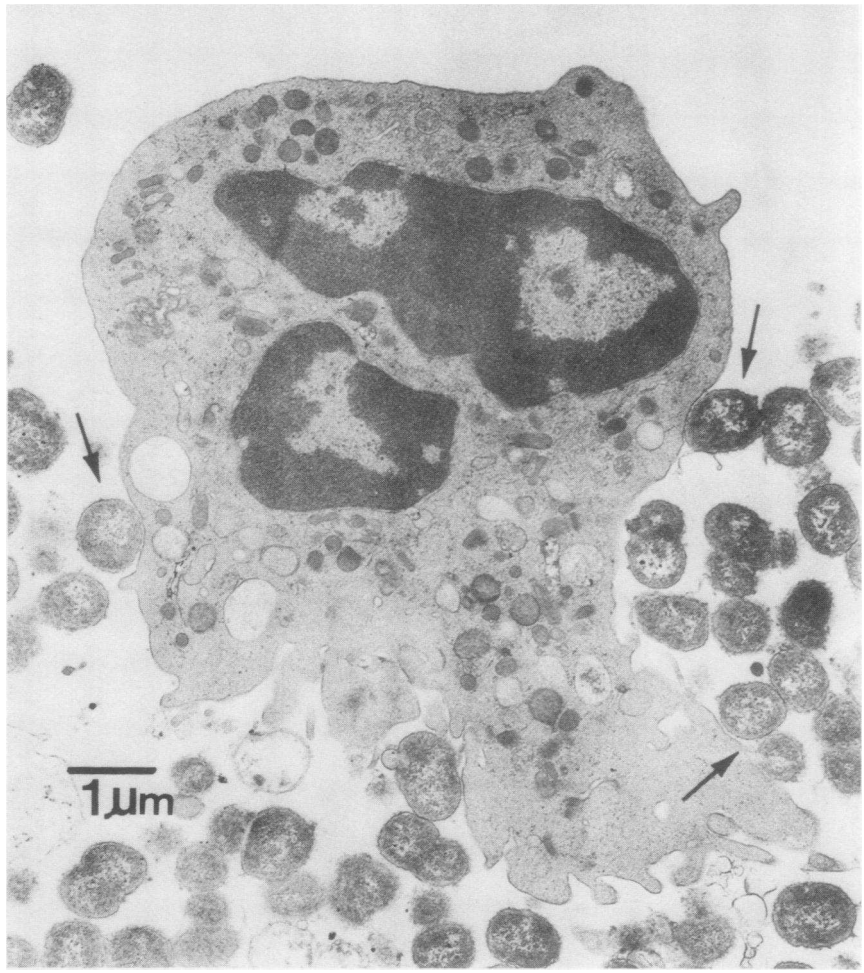

FIGURE 4 Electron microscopy of PMN-type I gonococcus interaction. Note the surface-associated organisms (arrows).

inating any early effect of differences in the cell association of gonococci.

Oxygen consumption. In the presence of serum oxygen consumption of PMN incubated with both type I gonococci and type III gonococci as well as with the heat-killed staphylococci is markedly increased when compared to PMN without bacteria (Table I). In the absence of serum both the type III gonococcus and staphylococci minimally stimulate PMN oxygen consumption. In contrast, marked oxygen consumption is observed with PMN incubated with type I gonococci in the absence of serum. The difference in PMN oxygen consumption induced by types I and III gonococci in the absence of serum is highly significant ($P < 0.0001$).

Hexose monophosphate shunt (HMP). In the presence of serum, PMN-HMP activity is stimulated by type I gonococci, type III gonococci, and staphylococci (Table I). In the absence of serum, the type I gonococcus causes marked HMP activity, but type III gonococci and staphylococci only minimally stimulate HMP activity. The greater stimulation of PMN-HMP in the absence of serum by type I gonococci compared to type III gonococci is highly significant ($P = 0.0009$).

Degranulation (Table II). PMN released significant quantities of lactoferrin, the specific granule marker, after interaction in serum with type I gonococci, type III gonococci, staphylococci, or zymosan. In the absence of serum, the lactoferrin release from PMN incubated with any of the stimulus particles was markedly reduced and was not significantly greater than lactoferrin release from PMN alone. Myeloperoxidase release into the media was significantly increased only by incubation with zymosan ($P = 0.03$).

In contrast to myeloperoxidase, a significant release of another primary granule marker, β-glucuronidase was induced by all stimulus particles in serum except type I gonococci (type III vs. type I, $P = 0.01$). In the absence of serum none of the particles stimulated sig-

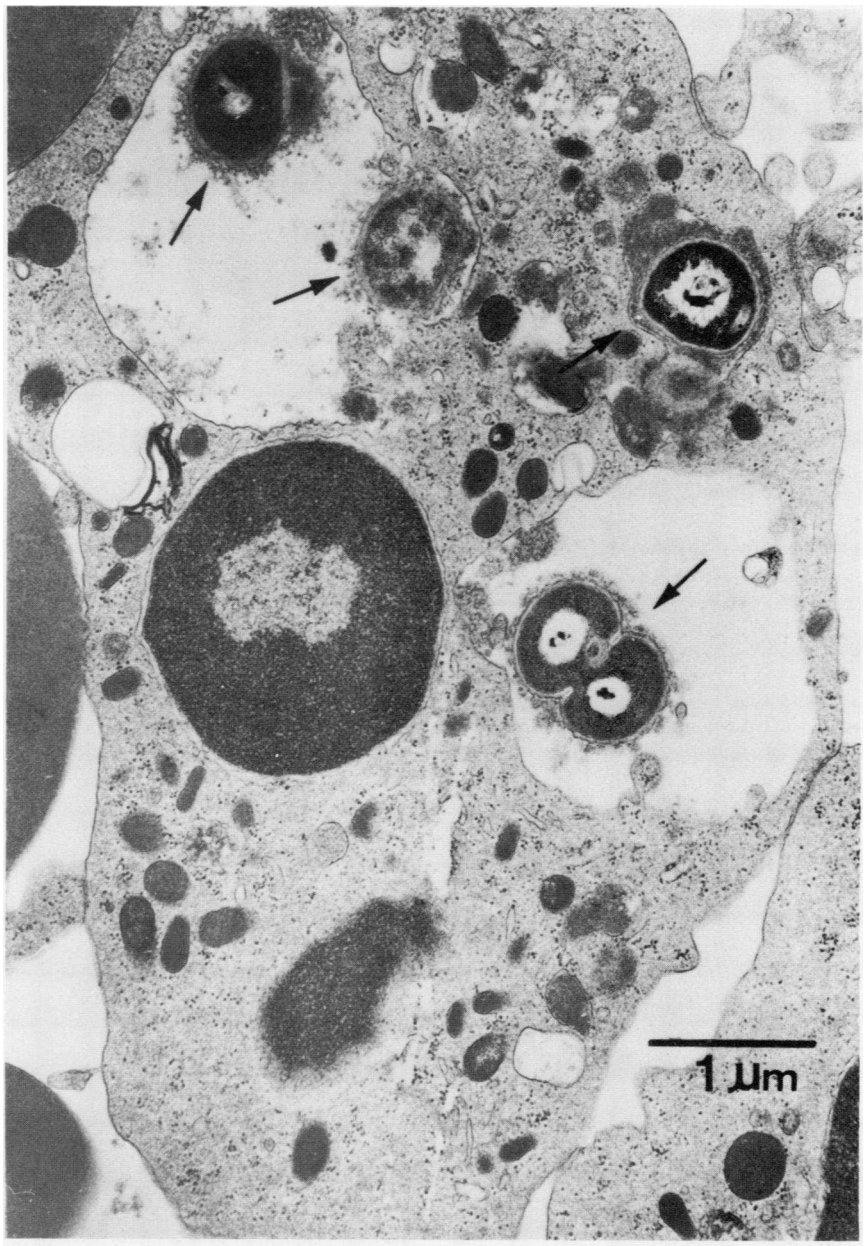

FIGURE 5 Electron microscopy of PMN-type III gonococcus interaction with serum. Note the extensive ingestion of the type III gonococci (arrows).

nificant β-glucuronidase release. β-glucuronidase release from stimulated PMN in the presence of CB and serum was similar. Attempts to measure lysozyme activity as an additional marker of specific granule release were unsuccessful because of interference by the gonococci with the lysozyme assay (Unpublished observations).

Myeloperoxidase-mediated iodination. In the presence of serum, PMN stimulated by the ingestion of type III gonococci or heat-killed staphylococci, iodinated protein (39,144±1,472 and 20,275±3,328 cpm, respectively) to a much greater extent than PMN stimulated by the attachment of type I gonococci (10,000±268 cpm; $P < 0.001$). In the absence of serum none of the organisms stimulated protein iodination by PMN and there was no significant difference in iodination with either type of gonococci.

Protein iodination in the presence of serum was decreased by CB for type I gonococci (1,786±152 cpm; $P < 0.001$) and type III gonococci (26,102±3,033 cpm;

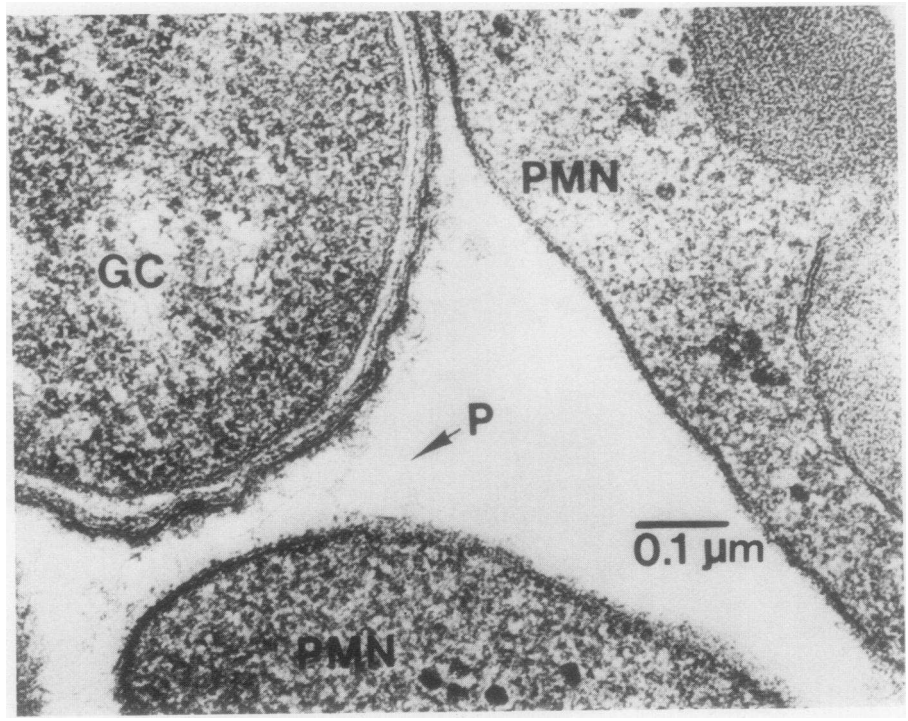

FIGURE 6 Electron microscopy of the PMN-type I gonococcus interaction. The type I gonococcus (GC) is closely opposed to the surface of the PMN. Gonococcal pili (P) can be seen in contact with the surface of a PMN pseudopod.

$P = 0.02$) but not for heat-killed staphylococci. Nevertheless, type III gonococci continued to stimulate significant iodination by PMN and the iodination was significantly greater than the iodination stimulated by type I gonococci ($P = 0.002$).

Cell-free peroxidase-mediated iodination. Lactoperoxidase-catalyzed iodination of type I gonococci and type III gonococci was similar 10,277±540 vs. 10,617±479 cpm, respectively; $P = 0.33$).

DISCUSSION

The mechanism by which virulent piliated gonococci escape ingestion is unknown but may relate to (*a*) the concomitant presence of an antiphagocytic capsule (27–29); (*b*) interference by pili or other surface structures with the sequential, circumferential interaction of opsonic ligands with their receptors on the PMN membrane which may be necessary for phagocytosis (30); or (*c*) a decrease in phagocytic membrane fluidity as a result of its interaction with the gonococcal pili (31).

Despite the failure of PMN to ingest piliated gonococci adherent to the PMN surface, there is a significant burst of PMN metabolism similar to that observed during ingestion of nonpiliated gonococci. In addition, the magnitude of the PMN metabolic response to piliated gonococci is similar regardless of the presence or absence of serum. These two facts strongly suggest that the mode of attachment of piliated and nonpiliated gonococci to PMN may be different and that differences in the outcome of the interaction of piliated and nonpiliated gonococci with PMN may be a direct consequence of these different modes of attachment. The

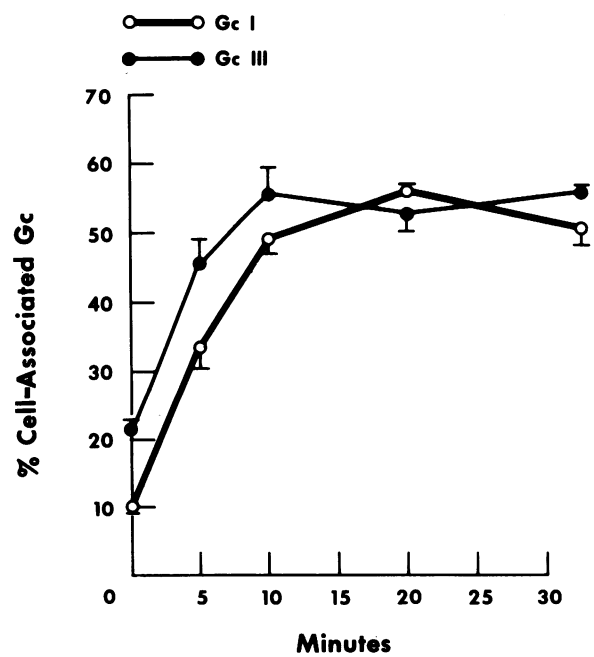

FIGURE 7 Uptake of radiolabeled gonococci (GC) by PMN.

TABLE I
Metabolic Responses of PMN to Gonococci

	PMN	PMN + Gc I	PMN + Gc III	PMN + Staphylococcus
Oxygen consumption, $\mu l\ O_2/h$*				
With serum	1.65±0.25	5.20±0.33	6.70±0.57	6.09±1.99
Without serum	0.52±0.04	4.29±0.26	1.55±0.12	0.52±0.04
HMP, *cpm**				
With serum	2,458±516	29,774±2,286	28,421±4,731	17,507±1,557
Without serum	703±119	23,856±3,032	7,475±859	1,717±179

Gc = gonococcus.
* Mean±SEM; n = a minimum of four separate experiments.

work of Goldstein et al. (32) lends support to this suggestion. These investigators demonstrated that opsonization of Sepharose beads (Pharmacia Fine Chemicals) with different immunologic ligands resulted in different degrees of stimulation of PMN oxidative metabolism and degranulation.

The finding that piliated organisms stimulate PMN metabolism in the absence of serum supports previous observations (33, 34) that membrane perturbation per se and not ingestion (32, 35–37) is the initiating event for the metabolic burst.

The results of the degranulation experiments demonstrate that in serum either attachment or ingestion can serve as a sufficient stimulus for the release of lactoferrin from the specific granule.

In contrast, we found that only zymosan, in serum, stimulated appearance of significant amounts of myeloperoxidase in the media compared to PMN alone. However, data from the iodination experiments indicate that myeloperoxidase is released into the phagosome during ingestion of type III gonococci because myeloperoxidase-mediated protein iodination increased markedly compared to PMN alone. In contrast, type I gonococci stimulated iodination poorly, suggesting that degranulation of the primary granule is not stimulated by the attachment of type I gonococci. Additional support is found in the observations that β-glucuronidase release was stimulated by type III gonococci but not by type I gonococci. Similar findings have recently been reported by Senff and Sawyer (38). The different results for primary granule release with the myeloperoxidase assay and the β-glucuronidase assay could reflect cell binding of myeloperoxidase (35) and subsequent diminished escape into the media despite primary granule release.

To further study the influence of attachment versus ingestion on the survival of nonpiliated gonococci, PMN were preincubated with CB, which prevents ingestion but not attachment. Preincubation of PMN with CB did not significantly alter the interaction of PMN with the virulent, piliated type I gonococci. However, CB dramatically improved the survival of type III gonococci without decreasing β-glucuronidase release. This suggests that the inability of PMN to kill attached type I gonococci is only partially explained by the failure of these organisms to stimulate the release of primary granule contents. The failure of CB-treated PMN to kill other attached organisms has been reported previously (39).

One possible explanation for the inability of CB-

TABLE II
PMN Degranulation Induced by Gonococci

	PMN	PMN + Gc I	PMN + Gc III	PMN + Staphylococcus	PMN + Zymosan
Lactoferrin, *μg/ml**					
With serum	16.4±4.8	41.1±12.6	45.0±15.1	39.7±11.2	42.0±12.2
Without serum	12.2±1.2	19.4±5.6	20.8±0.6	15.6±1.9	14.3±2.1
Myeloperoxidase, *μg/ml**					
With serum	4.8±1.0	6.2±2.2	8.7±1.8	10.4±2.3	12.9±3.6
Without serum	7.3±1.4	7.9±1.5	9.6±2.7	8.3±1.9	8.6±2.0
β-Glucuronidase, *Sigma units**					
With serum	62.1±6.0	78.6±13.8	132.0±9.0	155.5±45.5	195.9±22.2
Without serum	52.7±5.8	52.0±7.6	54.0±11.0	49.3±7.0	51.3±8.8
With serum and CB	56.4±4.8	71.1±7.5	110.4±24.0	107.0±31.6	167.1±13.2

Gc = gonococcus.
* Mean±SEM; n = a minimum of three separate experiments.

treated PMN to kill gonococci is the inhibition of phagosome formation by CB. This may result in the rapid dilution and diffusion of oxidative metabolites and granule constituents away from the PMN surface where they are formed and released. The phagocytic vacuole thus functions to contain these bactericidal components in a milieu at an optimal pH (40) for expression of their activity. It is possible that the near normal levels of protein iodination noted on stimulation of CB-treated PMN reflect iodination of serum and neutrophil proteins rather than bacterial protein because bacterial iodination is usually associated with bacterial death (25).

The failure of both normal PMN and CB-treated PMN to kill attached gonococci stands in marked contrast to the ability to PMN to kill organisms (6) or cells (7) that are too large to be ingested but to which the PMN are closely adherent. It is possible that the larger size of nonphagocytosable particles permits extensive and intimate contact between the PMN and the particle, in effect providing a closed space similar to a phagosome in which hydrogen peroxide, myeloperoxidase, and other antimicrobial factors can be contained.

The inability of normal PMN to kill virulent, attached type I gonococci appears to be the consequence of both inadequate release of enzymes from the PMN primary granules and the failure of PMN to incorporate type I gonococci in a phagocytic vacuole. Avirulent type III gonococci do stimulate normal primary granule release from CB-treated PMN but are not killed as they adhere to the PMN surface. The phagocytic vacuole thus plays a critical role in normal PMN bactericidal activity by providing a closed space in which the proper concentration of substances may be achieved at the proper pH to generate microbicidal activity.

ACKNOWLEDGMENTS

The authors are grateful for the expert technical assistance of Ms. Deborah Conard, Gail Sullivan, and Nancy Solomonsky and Mr. James Sullivan. We also are appreciative of the secretarial assistance of Ms. Lillian Robertson.

This work was supported in part by grants AI-09504 and AI-00069 from the National Institute of Allergy and Infectious Disease.

REFERENCES

1. Stossel, T. P. 1974. Phagocytosis. *N. Engl. J. Med.* **290**: 717–723, 773–780, 833–838.
2. Weissmann, G., R. B. Zurier, P. J. Spieler, and I. M. Goldstein. 1971. Mechanisms of lysosomal enzyme release from leukocytes exposed to immune complexes and other particles. *J. Exp. Med.* **134**: 149S–165S.
3. Henson, P. M. 1971. The immunologic release of constituents from neutrophil leukocytes. I. The role of antibody and complement on nonphagocytosable surfaces or phagocytosable particles. *J. Immunol.* **107**: 1535–1546.
4. Henson, P. M. 1971. The immunologic release of constituents from neutrophil leukocytes. II. Mechanisms of release during phagocytosis, and adherence to nonphagocytosable surfaces. *J. Immunol.* **107**: 1547–1555.
5. Root, R. K., J. Metcalf, N. Oshino, and B. Chance. 1975. H_2O_2 release from human granulocytes during phagocytosis. I. Documentation, quantitation, and some regulating factors. *J. Clin. Invest.* **55**: 945–955.
6. Diamond, R. D., R. Krzesicki, and W. Jao. 1978. Damage to pseudohyphal forms of *Candida albicans* by neutrophils in the absence of serum *in vitro*. *J. Clin. Invest.* **61**: 349–359.
7. Clark, R. A., and S. J. Klebanoff. 1975. Neutrophil-mediated tumor cell cytotoxicity: role of the peroxidase system. *J. Exp. Med.* **141**: 1442–1447.
8. Johnston, R. B., Jr., and J. E. Lehmeyer. 1976. Elaboration of toxic oxygen by products by neutrophils in a model of immune complex disease. *J. Clin. Invest.* **57**: 836–841.
9. Thongthai, C., and W. D. Sawyer. 1973. Studies on the virulence of *Neisseria gonorrhoeae*. I. Relation of colonial morphology and resistance to phagocytosis by polymorphonuclear leukocytes. *Infect. Immun.* **7**: 373–379.
10. Thomas, D. W., J. C. Hill, and F. J. Tyeryar, Jr. 1973. Interaction of gonococci with phagocytic leukocytes from men and mice. *Infect. Immun.* **8**: 98–104.
11. Ofek, I., E. H. Beachy, and A. L. Bisno. 1974. Resistance of *Neisseria gonorrhoeae* to phagocytosis: relationship to colonial morphology and surface pili. *J. Infect. Dis.* **129**: 310–316.
12. Dilworth, J. A., J. O. Hendley, and G. L. Mandell. 1975. Attachment and ingestion of gonococci by human neutrophils. *Infect. Immun.* **11**: 512–516.
13. Densen, P., M. F. Rein, J. A. Sullivan, and G. L. Mandell. 1978. Morphological observations of neutrophil-gonococcus interaction. *In* Immunobiology of *Neisseria gonorrhoeae*. American Society of Microbiology Publications Office, Washington, D. C. In press.
14. Swanson, J., S. J. Kraus, and E. C. Gotschlich. 1971. Studies on gonococcus infection. I. Pili and zones of adhesion: their relation to gonococcal growth patterns. *J. Exp. Med.* **134**: 286–906.
15. Swanson, J. 1973. Studies on gonococcus infection. IV. Pili: their role in attachment of gonococci to tissue culture cells. *J. Exp. Med.* **137**: 571–589.
16. Burton, K. 1956. A study of the conditions and mechanisms of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *Biochem. J.* **62**: 315–323.
17. Boyum, A. 1968. Isolation of mononuclear cells and granulocytes from human blood. *Scand. J. Clin. Lab. Invest.* **21** (Suppl. 97): 77–89.
18. Lee, T. J., J. Patterson, A. Schmoyer, W. Young, and P. F. Sparling. 1978. Familial deficiency of the sixth and seventh components of complement associated with bacteremic Neisseria infections. *In* Immunobiology of *Neisseria gonorrhoeae* American Society of Microbiology Publications Office, Washington, D. C. In press.
19. Petersen, B. H., J. A. Graham, and J. F. Brooks. 1976. Human deficiency of the eighth component of complement. The requirement of C8 for serum *Neisseria gonorrhoeae* bactericidal activity. *J. Clin. Invest.* **57**: 283–290.
20. Mandell, G. L., W. Rubin, and E. W. Hook. 1970. The effect of an HADH oxidase inhibitor (hydrocortisone) on polymorphonuclear leukocyte bactericidal activity. *J. Clin. Invest.* **49**: 1381–1388.
21. Mancini, G., A. O. Carbonara, and J. F. Heremans. 1965. Quantitation of antigens by single radial immunodifusion. *Immunochemistry.* **2**: 235–254.
22. Leffell, M. S., and J. K. Spitznagel. 1975. Fate of human

lactoferrin and myeloperoxidase in phagocytizing human neutrophils: effects of immunoglobulin G. subclasses and immune complexes coated on latex beads. *Infect. Immun.* **12:** 812–820.
23. Simmons, P. 1971. Quantitation of plasma proteins in low concentrations using RID. *Clin. Chim. Acta.* **35:** 53–57.
24. Fishman, W. H., K. Kato, C. L. Anstiss, and S. Green. 1967. Human serum β glucuronidase; its measurement and some of its properties. *Clin. Chim. Acta.* **15:** 435–447.
25. Klebanoff, S. J. 1962. Iodination of bacteria: a bactericidal mechanism. *J. Exp. Med.* **126:** 1063–1078.
26. Root, R. K., A. S. Rosenthal, and D. J. Balestra. 1972. Abnormal bactericidal, metabolic, and lysosomal functions of Chediak-Higashi Syndrome leukocytes. *J. Clin. Invest.* **51:** 649–665.
27. Hendley, J. O., K. R. Powell, R. Rodewald, H. H. Holzgrefe, and R. Lyles. 1977. Demonstration of a capsule on *Neisseria gonorrhoeae*. *N. Engl. J. Med.* **296:** 608–611.
28. Richardson, W. P., and J. C. Sadoff. 1977. Production of a capsule by *Neisseria gonorrhoeae*. *Infect. Immun.* **15:** 663–664.
29. James, J. F., and J. Swanson. 1977. The capsule of the gonococcus. *J. Exp. Med.* **145:** 1082–1086.
30. Griffin, F. M., J. A. Griffin, and S. C. Silverstein. 1975. Studies on the mechanism of phagocytosis. I. Requirements for circumferential attachment of particle-bound ligands to specific receptors on the macrophage plasma membrane. *J. Exp. Med.* **142:** 1263–1282.
31. Senff, L. M., W. D. Sawyer, and R. A. Haak. 1977. Effect of gonococci on nitroxide spin-labelled membranes of human blood cells. *In* Abstracts of the Annual Meeting of the American Society of Microbiology. American Society of Microbiology Publications Office, Washington, D. C. 77, D48.
32. Goldstein, I. M., H. B. Kaplan, A. Radin, and M. Frosch. 1976. Independent effects of IgG and complement upon human polymorphonuclear leukocyte function. *J. Immunol.* **117:** 1282–1287.
33. Zatti, M., and F. Rossi. 1967. Relationship between glycolysis and respiration in surfactant-treated leukocytes. *Biochim. Biophys. Acta.* **148:** 553–555.
34. Takanaka, K., and P. J. O'Brien. 1975. Mechanisms of H_2O_2 formation by leukocytes. Evidence for a plasma membrane location. *Arch. Biochem. Biophys.* **169:** 428–435.
35. Henson, P. M., and Z. G. Oades. 1975. Stimulation of human neutrophils by soluble and insoluble immunoglobulin aggregates. Secretion of granule constituents and increased oxidation of glucose. *J. Clin. Invest.* **56:** 1053–1061.
36. Goldstein, I. M., D. Roos, H. B. Kaplan, and G. Weissmann. 1975. Complement and immunoglobulins stimulate superoxide production by human leukocytes independently of phagocytosis. *J. Clin. Invest.* **56:** 1055–1063.
37. Roos, D., I. M. Goldstein, H. B. Kaplan, and G. Weissmann. 1976. Dissociation of phagocytosis, metabolic stimulation and lysosomal enzyme release in human leukocytes. *Agents Actions.* **6:** 256–259.
38. Senff, L. M., and W. D. Sawyer. 1977. Release of enzymes from human leucocytes during incubation with *Neisseria gonorrhoeae*. *Br. J. Vener. Dis.* **53:** 360–363.
39. Okuda, K. 1975. Effects of cytochalasin B. on the intracellular bactericidal activity of human neutrophils. *Antimicrob. Agents Chemother.* **7:** 736–741.
40. Mandell, G. L. 1970. Intraphagosomal pH of human polymorphonuclear neutrophils. *Proc. Soc. Exp. Biol. Med.* **134:** 447–449.