THE INTERACTION IN VITRO BETWEEN HUMAN POLYMORPHONUCLEAR LEUKOCYTES AND *NEISSERIA GONORRHOEAE* CULTIVATED IN THE CHICK EMBRYO*

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Gonococcal infections are a serious public health problem. Despite renewed public health efforts, the incidence of gonorrhea has continued to rise 10–15% annually since 1967 (1). Examination of clinical specimens from infected patients often reveals gonococci closely associated with polymorphonuclear leukocytes. Despite this long-standing clinical observation, factors related to gonococci-human leukocyte interactions have remained undefined. This has been in large part due to the lack of an experimental animal and a suitable method for the laboratory cultivation of these organisms.

Kellogg and co-workers described colonial variation of gonococci and based on direct inoculation of organisms into the male urethra correlated type 1 and 2 organisms with virulence and type 3 and 4 gonococci with avirulence. Nonselective transfer of type 1 and 2 gonococci on artificial medium resulted in reversion to type 3 and 4 organisms (2, 3). Recent morphologic studies have suggested that colonial types 1 and 2 gonococci are more resistant to phagocytosis than type 3 and 4 organisms (4–6).

This report describes a method for the laboratory cultivation of gonococci which allows for quantitative in vitro phagocytosis studies of viable organisms. The findings indicate that log-phase virulent gonococci possess antiphagocytic surface factors and that avirulent organisms are ingested and rapidly killed by human polymorphonuclear leukocytes. Heat-labile serum factors are necessary for phagocytosis of log-phase organisms. In addition, log-phase virulent gonococci attach to the surface membrane but are not interiorized by human polymorphonuclear leukocytes in the presence or absence of fresh human serum.

Materials and Methods

Bacteria. Strains of *Neisseria gonorrhoeae* (strains 44, 49, 51, 54, 60, and 65) were isolated from the blood, synovial fluid, and cervix of patients with disseminated gonococcemia. All isolates were identified by gram stain, oxidase reaction, and sugar fermentations. After one to three subcultures on GC agar base (Difco Laboratories, Detroit, Mich.) supplemented with 1% IsoVitaleX (Baltimore Biological Laboratories, Cockeysville, Md.) and incubated overnight at 36°C in 5% CO_2 atmosphere (National Incubator, Portland, Oreg.), isolates were suspended in 1 ml 1% proteose peptone broth (Difco Laboratories) with 20% sterile glycerol (J. T. Baker Chemical Co., Phillipsburg, N. J.) and preserved at −65°C (Revco Ultra-Low Temperature Unit; Revco, Inc., West Columbia, S. C.), in 2 ml Nunc serum tubes (Vanguard International, Inc., Red Bank, N. J.).

The strain of coagulase-negative *Staphylococcus epidermidis* employed as a positive control in all phagocytosis experiments was maintained on Trypticase Soy Agar with 5% defibrinated sheep blood (Baltimore Biological Laboratories) or in Trypticase Soy Broth (Baltimore Biological Laboratories). Before each phagocytosis test, 5 ml of broth was inoculated with an overnight agar culture and incubated aerobically at 36°C. After 18 h, 0.09 ml of the stationary broth culture was placed in 2 ml of diluent. The final concentration of organisms in the diluent was 2–3 × 10^8 colony-forming units (cfu)[1] per ml.

The maintenance of stock and daily cultures and the preparation of group C meningococcal suspensions for phagocytosis studies have been previously described (7, 8).

Cultivation of Gonococci in the Chick Embryo. Type 1 and type 2 or type 4 colonies were cloned from 18-h agar cultures and suspended in 1% proteose peptone broth with sterile swabs, MH 100 culture system with Amies clear transport media (Precision Dynamics Corp., Burbank, Calif.). 0.2 ml of the suspension was inoculated into the allantoic cavity of 10-day chick embryos (White Leghorn chickens, Shamrock Farms, New Brunswick, N. J.). Inoculation was performed as follows: After delineating by transillumination the location of the allantoic cavity and an area devoid of blood vessels, the egg shell was cleansed with iodine and then alcohol which was allowed to evaporate. A hole was made with a 20 gauge needle through which a 25 gauge needle attached to a 1 ml tuberculin syringe was inserted. After inoculation, the hole was covered with cellophane tape. Organisms were harvested in the following manner: The tape was cleansed with alcohol, and allantoic fluid removed through the same hole with a 20 gauge needle attached to a 5-ml syringe. Care was taken not to enter the yolk sac nor to cause blood vessel rupture. Approximately 4–5 ml allantoic fluid could be removed from 10-day chick embryos. If fluid was difficult to remove by needle aspiration, the shell overlying the air sac was removed and fluid aspirated with a 5-ml pipette.

Before phagocytosis studies, all gonococcal isolates were cultivated daily for at least 10 days in chick embryos incubated aerobically and stationary at 36°C. After the last serial passage, infected allantoic fluid was cultured on agar for 18 h. Colonies were removed with swabs, suspended in 1 ml proteose peptone broth containing 20% glycerol and frozen at −65°C (stock cultures). The viability of gonococci was not affected significantly by freezing organisms in this medium for 6–12 mo. The day before each phagocytosis experiment, a stock culture was thawed and 0.1 ml was inoculated into the allantoic cavity of two embryos. After 18 h of aerobic, stationary incubation at 36°C, allantoisc fluid containing 3–5 × 10^8 cfu/ml was withdrawn from each embryo. 0.2 ml (approximately 1–3 × 10^7 cfu/ml) was inoculated into each of four embryos which were placed on an Eberbach reciprocating shaker (Eberbach Corp., Ann Arbor, Mich.) (90 oscillations/min) and incubated aerobically at 36°C. Allantoic fluid was removed after 4.5 h (log-phase organisms) and 18 h (stationary-phase organisms) of incubation and centrifuged at 450 rpm for 5 min to remove chick embryo cells. Gonococci were not sedimented by this low-speed centrifugation.

Serum. Fresh normal human sera were obtained from donors who denied previous gonococcal infections and did not have an elevated antipili antibody titer by radioimmunoassay (courtesy of Dr. T. Buchanan, The Rockefeller University, New York). All sera were collected and used either the same day or within 2 wk after freezing in 1-ml aliquots at −15°C. Fresh human sera were frozen once and thawed immediately before each experiment. Normal sera were also heat inactivated at 56°C for 30 min.

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[1] *Abbreviations used in this paper:* cfu, colony-forming units; PMN, polymorphonuclear.

Leukocytes. Human leukocyte suspensions were prepared from blood drawn from two volunteers who had no previous history of gonorrhea nor elevated antipili antibody titers. Blood was mixed with an equal volume of physiologic saline containing 2% Dextran 250 (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden) and 0.02% heparin (Connaught Medical Research Laboratories, Toronto, Canada). Tubes with this mixture were slanted at 45° for 15 min at room temperature to allow for red cell sedimentation. The leukocyte-rich plasma was then decanted and the volume and granulocyte count determined. Leukocytes were sedimented at 1,000 rpm for 10 min at room temperature, washed first in heparinized saline and then in saline alone, and resuspended to the desired concentration in sterile Gey's balanced salt solution (Microbiological Associates, Inc., Bethesda, Md.) with 0.1% gelatin (Difco Laboratories, Inc.). This diluent is hereafter designated gel-Gey's medium.

Preparation of Phagocytosis Test. The phagocytosis test employed in these studies was a modification of that described by Maaloe and others (7, 9). However, because of the decrease in survival of gonococci when glassware was used and when organisms were incubated for 120 min, phagocytosis tests were performed in 10×78-mm plastic tubes with snap caps (Falcon Plastics, Div. of BioQuest, Los Angeles, Calif.) and for an incubation period of 60 min. To each tube was added 0.8 ml leukocyte suspension, 0.1 ml bacterial suspension, and 0.1 ml of normal human serum; thus, the total vol was 1.0 ml and contained approximately 2.0×10^7 leukocytes and bacteria (bacteria:leukocyte ratio, 1:1) and 10% serum. Serum was replaced with gel-Gey's medium in some experiments. Tubes were sealed and tumbled end over end (Spinerette, New Brunswick Scientific Co., Inc., New Brunswick, N. J.) at a 45° angle for 60 min aerobically at 36°C. Control tubes were employed in each phagocytosis experiment: A serum control without leukocytes to rule out the bactericidal effect of serum alone, and a positive phagocytosis control employing *S. epidermidis*, human leukocytes, and fresh normal human serum to ensure that each leukocyte preparation was capable of efficient phagocytosis. The viability of gonococcal strains in 10% fresh normal human serum was first tested in a similar manner as that for serum controls.

The technique for the enumeration of viable gonococci was a modification of that used previously in this laboratory (7, 8). To determine the total number of viable bacteria a 0.003-ml aliquot was removed with a standardized platinum loop at time intervals of 0, 30, and 60 min and placed in a 1.0 ml of distilled water, pH 7.4, containing 0.01% crystallized bovine plasma albumin (Armour Pharmaceutical Co., Chicago, Ill.). The suspension was vortexed, serially diluted in gel-Gey's medium, and 0.1 ml of the dilutions spread on the surface of GC agar (total count). Simultaneously, a 0.003-ml aliquot was removed and placed in 1.0 ml of gel-Gey's medium. After thorough mixing, the suspension was centrifuged at 450 rpm for 5 min at room temperature and 0.1 ml of supernatant fluid removed, serially diluted to gel-Gey's medium, and agar plated (extracellular count). Gonococci were not sedimented under these conditions. Sedimented leukocytes were then resuspended in 1.0 ml distilled water with 0.01% bovine plasma albumin, mixed thoroughly, serially diluted in gel-Gey's medium, and agar plated (cell-associated count). Bacterial suspensions in the serum control (*Gonococci*) and positive phagocytosis (*S. epidermidis*) tubes were diluted in gel-Gey's medium and distilled water with albumin, respectively, and agar plated. GC agar plates were incubated at 36°C in the presence of moisture and 5% CO_2, and bacterial colonies (cfu) counted after 18 h. A phagocytosis test was considered positive when greater than 90% of organisms were ingested and killed after 60 min of incubation.

Results

Cultivation of Gonococci in the Chick Embryo

Cultivation of the gonococcus in the allantoic cavity of 10-day chick embryos provided the following important properties necessary for subsequent quantitative in vitro phagocytosis studies of viable gonococci: (a) log-phase growth, (b) resistance to killing by fresh human serum, (c) maintenance of viability in medium constituents, (d) stability of colonial types, and (e) absence of clumping.

Log-phase growth of gonococci. The growth curves for strain 54 (type 1) in

the allantoic cavity of 10-day chick embryos, shaking and stationary, are demonstrated in Fig. 1. The solid and dotted lines represent the mean number of viable organisms from four embryos. A similar pattern of growth was observed for all strains and colonial types after 10 daily passages in the allantoic cavity. Cultivation of gonococci in shaking embryos resulted in a lag phase of growth for 1 h, log growth for 5–7 h, and a stationary growth phase for an additional 10 or more h. The generation time during the rapid multiplication period was 72 min. Log-phase growth in stationary embryos was less than that in shaking embryos.
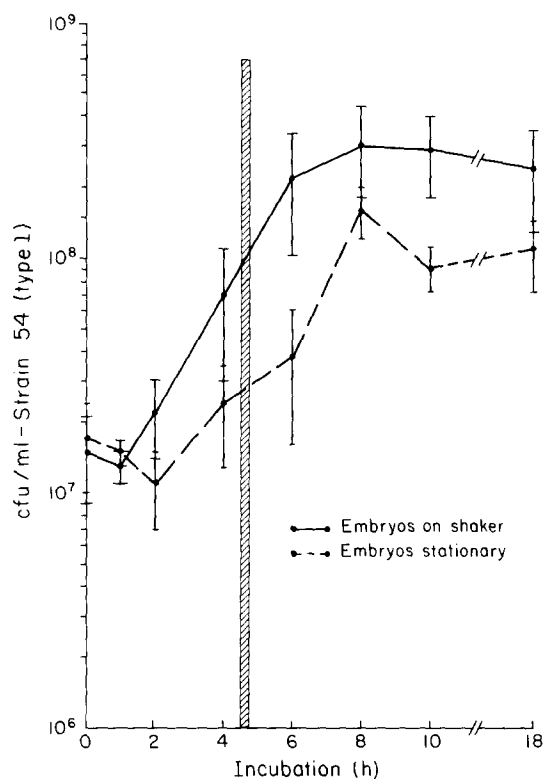

Fig. 1. The growth curve of a representative gonococcal strain in the allantoic cavity of 10-day chick embryos. The solid and dotted lines represent the mean number of viable organisms from four embryos, shaking and stationary, respectively. All embryos were incubated aerobically at 36°C.

Similar findings were observed in allantoic fluid removed from the chick embryo. During the cultivation period of 24 h, death of embryos was never observed. Examination of the chorioallantoic membrane with hematoxylin and eosin and gram stains after 5 and 18 h of gonococcal cultivation failed to reveal inflammatory cells or organisms within the membrane. For phagocytosis studies employing log-phase organisms, fluid was harvested after 4.5-h cultivation (as demonstrated by the shaded vertical bar); for stationary-phase organisms, after 18-h cultivation.

VIABILITY OF GONOCOCCI IN NORMAL HUMAN SERUM. Gonococci cultivated on

artificial media are susceptible to the bactericidal effect of fresh serum (10). Since quantitative phagocytosis studies of viable gonococci rely on the survival of these organisms in fresh serum, the viability of each strain and colonial type was determined. Results of the bactericidal effect of 10% fresh normal human serum (the concentration employed in phagocytosis studies) on five strains and three colonial types in the log phase of growth are shown in Table I. Log-phase organisms survived in this serum concentration over an incubation period of 60 min. In addition, some strains in the log phase survived in up to 50% fresh normal human serum. Many strains in the stationary phase of growth, however, were susceptible to the cidal effect of 10% fresh serum. These findings are also shown in Table I. Whereas four T-1 and T-2 strains (strains 65, 54, 51, and 49) in the log phase of growth resisted the cidal effect of serum, these same strains were killed by fresh serum when in the stationary phase of growth, i.e., a onefold to ninefold decrease in the number of viable bacteria. Heat inactivation of fresh normal

TABLE I

Viability of Gonococci in 10% Fresh Normal Human Serum

Log-phase gonococci			Stationary-phase gonococci		
Strain (colony type)	cfu/ml after incubation at 36°C		Strain (colony type)	cfu/ml after incubation at 36°C	
	0 min	60 min		0 min	60 min
65 (T-1)	6.6×10^6	8.0×10^6	65 (T-1)	2.8×10^7	4.0×10^6
54 (T-1)	1.3×10^7	1.6×10^7	54 (T-1)	3.3×10^7	2.8×10^6
51 (T-2)	2.3×10^6	2.0×10^6	51 (T-2)	9.0×10^6	8.0×10^6
49 (T-2)	2.1×10^7	1.6×10^7	49 (T-2)	5.3×10^7	2.7×10^7
44 (T-4)	1.1×10^7	1.7×10^7			
54 (T-4)	2.0×10^7	1.1×10^7			

serum eliminated the cidal effect. Based on these findings, a limited number of strains in the stationary phase of growth were employed in phagocytosis studies.

VIABILITY OF GONOCOCCI IN MEDIUM CONSTITUENTS. To ensure that gonococci remained viable during the incubation period of phagocytosis tests, the survival of all gonococcal strains and colonial types was first examined in medium constituents. Results of a representative strain are shown in Table II. Type 1 and type 4 organisms survived in gel-Gey's medium for at least 60 min, the diluent employed in phagocytosis studies. Gonococci also survived in distilled water with 0.01% albumin for 30 min. This diluent was used to lyse leukocytes and the period of incubation never exceeded 15 min.

STABILITY OF COLONIAL TYPES. Since previous studies suggest that type 1 and 2 gonococci are more resistant to phagocytosis than type 3 and 4 organisms, a system for the laboratory cultivation of gonococci must provide a population of organisms with the same colonial type. Virulent gonococci cultivated in biphasic media or broth revert to avirulent colonial types (11). However, cloned type 1 and 2 or type 4 gonococci from agar or frozen cultures remained the same type during repeated cultivation in the allantoic cavity of chick embryos. Before and after

TABLE II

Viability of Log-Phase Gonococcal Strain 54 in Medium Constituents

Colony type	Medium	cfu/ml after incubation at 36°C		
		0 min	30 min	60 min
Type 1	Gel-Gey's*	3.6×10^6	4.1×10^6	4.8×10^6
	Alb-H$_2$O‡	3.7×10^6	3.5×10^6	—
Type 4	Gel-Gey's	3.3×10^6	3.0×10^6	2.8×10^6
	Alb-H$_2$O	3.3×10^6	3.6×10^6	—

* Gey's balanced salt solution + 0.1% gelatin.
‡ Sterile distilled H$_2$O + 0.01% albumin.

each in vitro phagocytosis experiment, organisms harvested from the embryo were also subcultured on agar to document stability of colonial type; greater than 95% of gonococci remained the same type.

ABSENCE OF BACTERIAL CLUMPING. Type 1 and 2 gonococci clump when grown in agar or in biphasic media or broth (12). Since phagocytosis was measured quantitatively by the number of viable bacteria (cfu) remaining in the total, extracellular and cell-associated compartments, clumping of organisms would preclude the use of this technique. Type 1 and 2 or type 4 Gonococci did not clump during cultivation in the allantoic cavity. However, some strains subsequently agglutinated when incubated in 10% fresh normal human serum for 60 min; these strains were omitted from phagocytosis studies.

Gonococci-Human Polymorphonuclear Leukocyte Interactions

THE INTERACTION OF LOG-PHASE TYPE 1 AND TYPE 2 GONOCOCCI WITH HUMAN POLYMORPHONUCLEAR LEUKOCYTES. Previous studies of gonococci have employed stained smears for evaluation of phagocytosis (4-6). Fresh serum was also omitted from these studies. Previous attempts to use the Maaloe technique have been unsuccessful because of bacterial clumping and instability of colonial types (5). Cultivation of gonococci in the allantoic cavity of 10-day chick embryos ensured those properties necessary for quantitative assessment of the phagocytosis of viable gonococci. The interactions of two representative log-phase type 1 and type 2 strains with human polymorphonuclear (PMN) leukocytes in the presence of 10% fresh and heat-inactivated normal human serum and in the absence of serum are shown in Fig. 2 and Fig. 3. The total count, cell-associated count, and serum control remained essentially the same under all three experimental conditions during the incubation period of 60 min indicating that phagocytosis did not occur. However, when the extracellular counts were examined, two patterns of gonococci-leukocyte interaction were observed. Either the extracellular count remained the same, as shown in Fig. 2, or more commonly, the number of viable organisms in the extracellular compartment decreased 8- to 12-fold during the period of incubation (Fig. 3). Clumping of organisms was not observed by microscopy. The decrease in the extracellular count suggested that gonococci were cell-associated, i.e., within leukocytes or
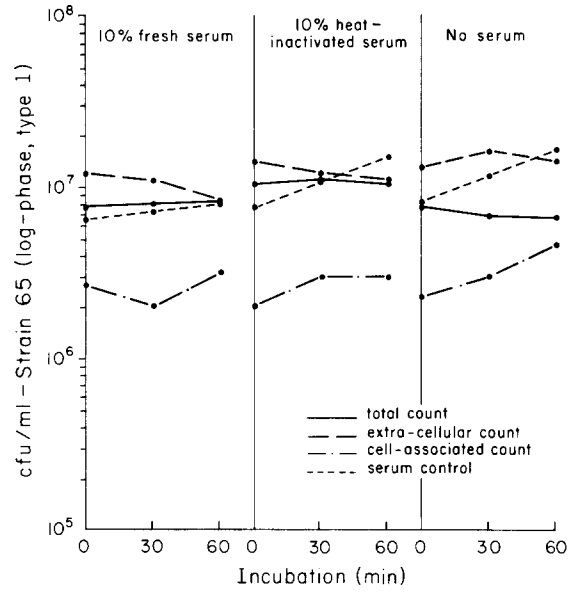
FIG. 2. The interaction between gonococcal strain 65 (log phase, type 1) and human PMN leukocytes in the presence or absence of fresh and heat-inactivated human serum.
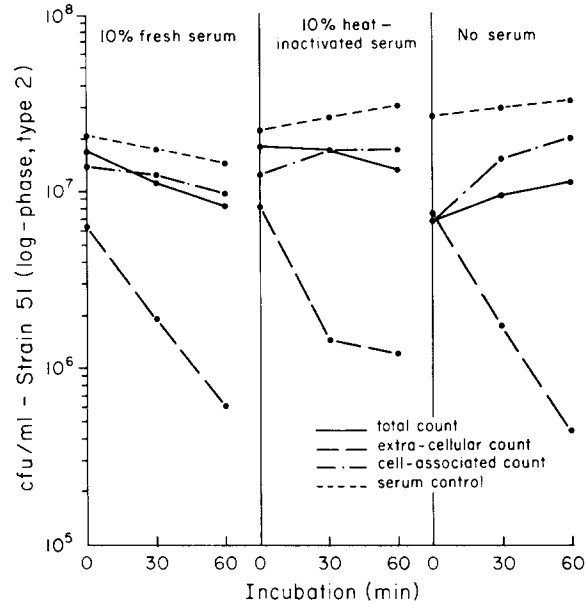

FIG. 3. The interaction between gonococcal strain 51 (log phase, type 2) and human PMN leukocytes in the presence or absence of fresh and heat-inactivated human serum.

attached to the surface membrane. Studies were therefore designed to examine these two possibilities.

The susceptibility of the six gonococcal strains to penicillin G was determined by the agar dilution method. Potassium Penicillin G Crystals (Eli Lily & Co.,

Indianapolis, Ind.) were dissolved in sterile distilled water (stock solutions). The stock solutions were added to sterilized GC agar containing 1% IsoVitaleX to achieve a final concentration of penicillin G of 0.05–1.0 μg/ml. A 24-h agar culture of gonococci was suspended in proteose peptone broth. This suspension was further diluted 1:1,000 and 0.2 ml placed on the surface of agar plates containing penicillin G. After incubation at 36°C in 5% CO_2 for 48 h, the presence or absence of growth was recorded. All strains were susceptible to 0.8 μg/ml or less of penicillin G.

The effect of penicillin G on the cell-associated count was examined employing a modification of the methods described by Holmes et al. and by Watt (13, 14). After the 60-min incubation period of the phagocytosis test, the bacteria-leukocyte-serum mixture was centrifuged at 800 rpm for 10 min. The supernate was decanted and the pellet resuspended in 1 μg/ml penicillin G. At this concentration, penicillin G does not enter human PMN leukocytes (15). The suspension was incubated at 36°C in a shaking water bath (American Optical Corp., Chicago, Ill.) and samples removed at 0, 60, and 120 min with a 0.003 ml calibrated loop. The sample was washed twice with gel-Gey's medium, resuspended in 1.0 ml of distilled water containing 0.01% bovine albumin, serially diluted in gel-Gey's medium, and agar plated. Control samples without penicillin G were included in each experiment.

All viable gonococci in the cell-associated compartment were killed after 120 min in the presence of 1 μg/ml penicillin G. Viable counts of gonococci remained the same in control samples without penicillin G. These findings suggested that leukocyte-associated gonococci were extracellular.

THE INTERACTION OF STATIONARY-PHASE TYPE 1 AND TYPE 2 GONOCOCCI WITH HUMAN PMN LEUKOCYTES. Gonococci harvested after 18 h of cultivation in the allantoic cavity of the chick embryo are in the stationary phase of growth. As shown in Fig. 4, the interaction of these organisms with leukocytes differed markedly from that when log-phase gonococci were employed. During the incubation period of 60 min, the total and extracellular counts decreased 10-fold in the presence of 10% fresh human serum suggesting that these organisms were ingested and rapidly killed by human PMN leukocytes. In the presence of 10% heat-inactivated serum and in the absence of serum a decrease of 70–80% in the total and extracellular counts was also observed. As mentioned previously, a 1-fold decrease of viable organisms in the fresh normal serum control without leukocytes was seen. These findings emphasize the necessity to use log-phase gonococci for the study of surface factors which may confer resistance to phagocytosis.

THE INTERACTION OF LOG-PHASE TYPE 4 GONOCOCCI WITH HUMAN PMN LEUKO-CYTES. As shown in Fig. 5, a one to two log decrease in the total, extracellular, and cell-associated counts was observed only in the presence of 10% fresh normal serum indicating that organisms were ingested and rapidly killed by PMN leukocytes. Viable counts remained the same or slightly increased in the presence of 10% heat-inactivated serum and in the absence of serum. Log-phase type 4 organisms in control tubes without leukocytes remained viable in the presence or absence of serum. These findings demonstrate that the ingestion of log-phase gonococci by human PMN leukocytes requires heat-labile serum factors.
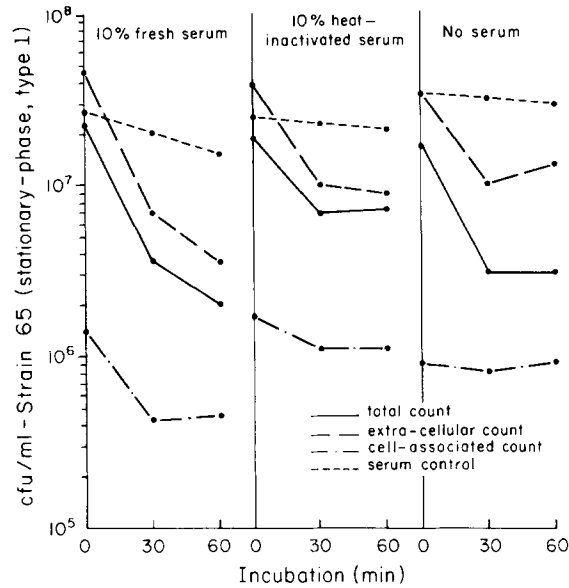
Fig. 4. The interaction between gonococcal strain 65 (stationary phase, type 1) and human PMN leukocytes in the presence or absence of fresh and heat-inactivated human serum.
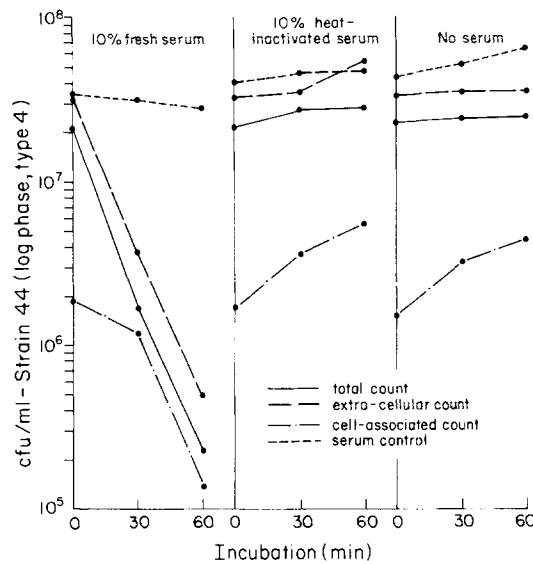

Fig. 5. The interaction of gonococcal strain 44 (log phase, type 4) and human PMN leukocytes in the presence or absence of fresh and heat-inactivated serum.

Attachment of Gonococci to Human PMN Leukocyte Surface Membrane.
Morphologic studies of gonococci-human PMN leukocyte interactions were performed in order to investigate further cell-associated organisms described in previous experiments.

Human PMN leukocytes were obtained by adding 5 ml of blood to an equal

volume of 2% Dextran 250 containing 0.02% heparin. Tubes containing the mixture were slanted at 45° for 20 min at room temperature to allow for red cell sedimentation. 0.3 ml of the leukocyte-rich plasma containing approximately 1 × 10^6 PMN leukocytes was placed on the surface of 22-mm square cover slips (Corning, Medford, Mass.) which had been immersed previously in 95% ethanol, flamed, and placed in sterile 35 × 10-mm plastic tissue culture dishes (Falcon Plastics, Div. of BioQuest). The cover slips were incubated for 30 min at 36°C in 5% CO_2 allowing the leukocytes to adhere in an even monolayer to the glass surface. The cover slips were then washed vigorously with Gey's balanced salt solution to remove all nonadhering cells. Each cover slip was overlaid with 0.9 ml allantoic fluid containing 1-2 × 10^8 viable gonococci and 0.1 ml of fresh or heat-inactivated human serum. The bacteria-PMN leukocyte ratio was approximately 100:1. After incubation for an additional 30 min at 36°C in 5% CO_2, the fluid was removed by aspiration and 1.5 ml of 2.5% glutaraldehyde (Fisher Scientific Co., Fair Lawn, N. J.) in 0.1 M sodium cacodylate (pH 7.4) was added for an additional 18 h at 4°C. Cover slips were then inverted onto a drop of water on a glass slide, attached with parafin, and observed by phase-contrast microscopy and Nomarski differential interference-contrast microscopy. Photographs were taken on Kodak Panatomic-X film.

Employing phase-contrast optics, the interaction of log-phase type 1 and 2 gonococci with human PMN leukocytes in the presence of 10% fresh normal human serum is illustrated in Fig. 6 a. Organisms were attached or adherent to the surface membrane and could not be seen free in the extracellular medium. PMN leukocytes remained well spread on the glass and lysosomal granules and the multilobed nucleus were easily seen. Phagocytic vacuoles and ingested organisms were not observed. As shown in Figure 7 a similar findings were observed when preparations of log-phase type 1 and 2 organisms and leukocytes were examined by interference-contrast microscopy. The interaction of log-phase type 4 organisms with PMN leukocytes in the presence of 10% fresh human serum is illustrated in Figs. 6 b and 7 b. Phase-contrast microscopy (Fig. 6 b) and interference-contrast microscopy (Fig. 7 b) revealed PMN leukocytes which were rounded up and contained many phagocytic vacuoles. Organisms were frequently observed on the surface membrane and within vacuoles. The morphologic studies of gonococci-leukocyte interactions are summarized in Table III. Log-phase type 1 and 2 gonococci attached to the surface membrane of PMN leukocyte but were not ingested in the presence of 10% fresh or heat-inactivated human serum. Stationary-phase type 1 and 2 gonococci attached to the surface membrane in the presence of either fresh or heat-inactivated serum. Ingestion occurred more readily when fresh serum was present in the system. Log-phase type 4 gonococci attached to the surface membrane under both experimental conditions. Ingestion however was only observed in the presence of fresh human serum. These morphologic observations confirm studies employing the Maaloe technique. To ensure that allantoic fluid at the concentration employed in these studies did not influence attachment or ingestion, log-phase group C meningococci and *S. epidermidis* were suspended in fresh allantoic fluid and added to monolayers of PMN leukocytes in the presence of 10% fresh human serum. Meningococci suspended in either gel-Gey's medium or allantoic fluid did not attach to the
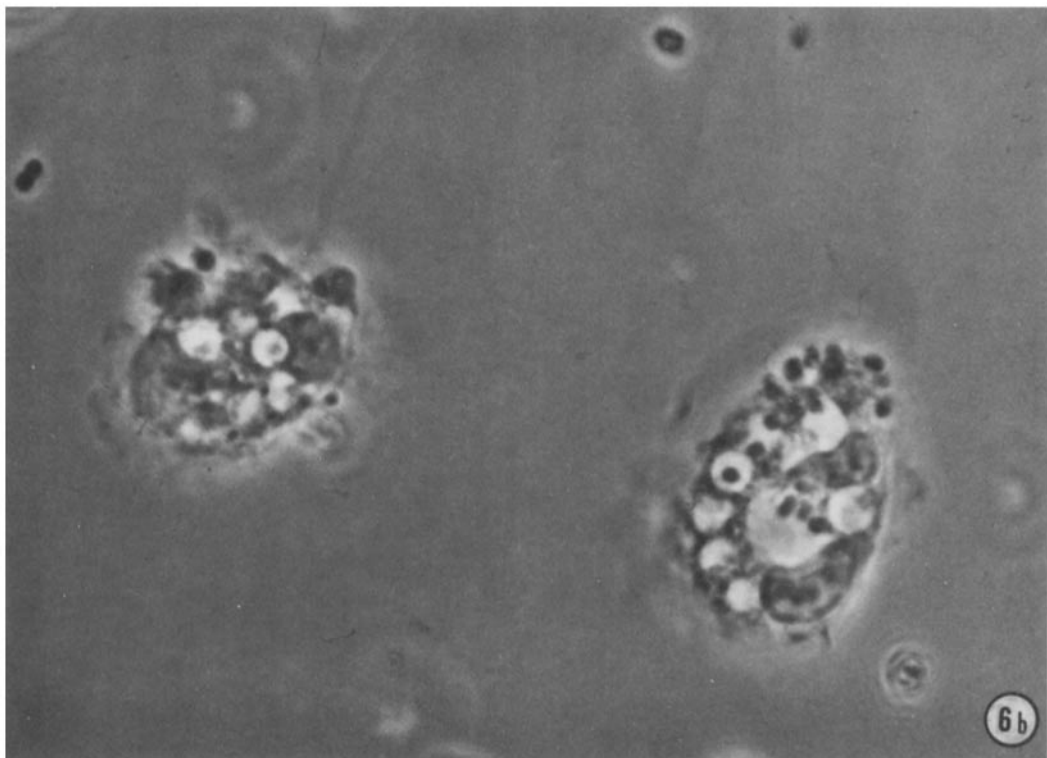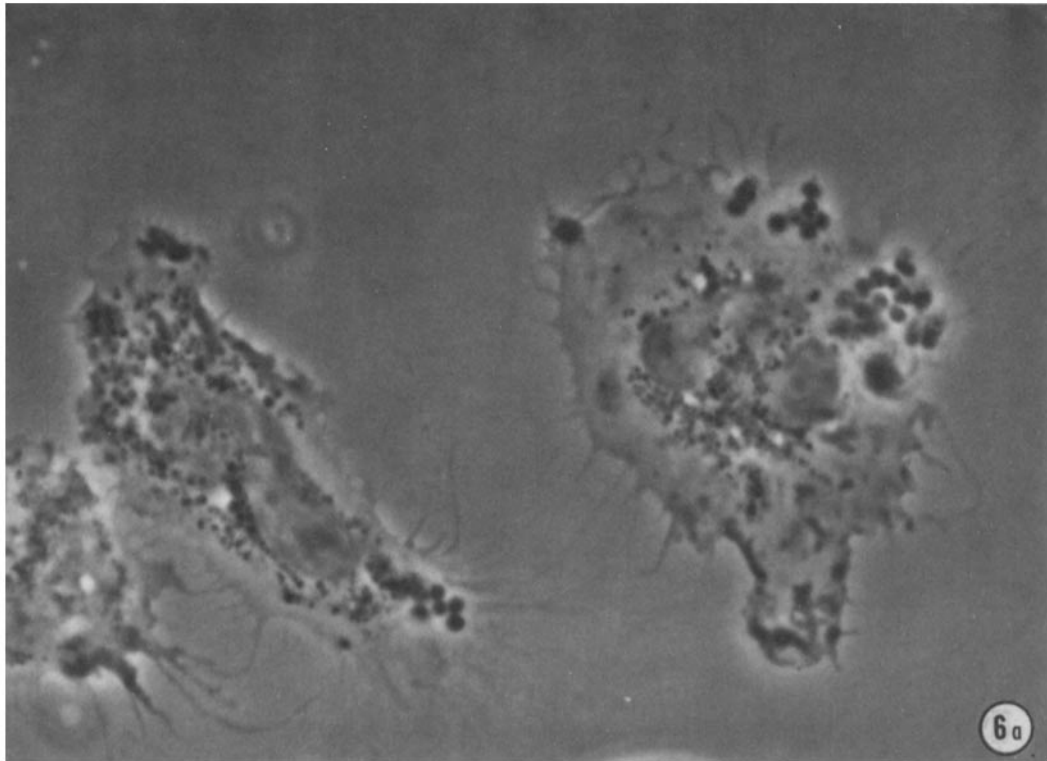
FIG. 6. (a) The interaction between log-phase type 1 and 2 gonococci and human PMN leukocytes incubated for 30 min in the presence of fresh serum. Organisms are attached to the surface membrane and are not seen in the extracellular medium. Leukocytes remain well spread on the glass surface and cytoplasmic granules appear intact. Phase-contrast microscopy, × 1,250. (b) The interaction between log-phase type 4 gonococci and human PMN leukocytes incubated for 30 min in the presence of fresh human serum. Organisms are attached to the surface membrane and are seen within large phagocytic vacuoles. Leukocytes are rounded up and extensive degranulation has occurred. Phase-contrast microscopy, × 1,250.
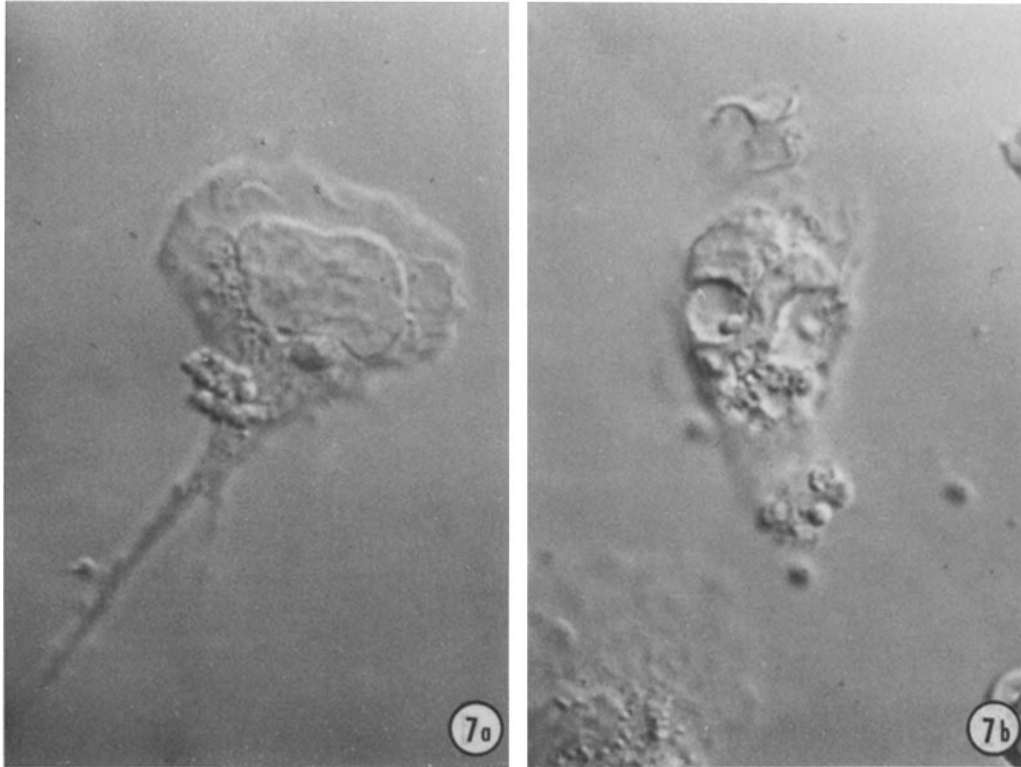
FIG. 7. (a) The interaction between log-phase type 1 and 2 gonococci and human PMN leukocytes incubated for 30 min in the presence of fresh human serum. Organisms are attached to the surface membrane. The leukocyte is well spread revealing the extended pseudopod, multilobed nucleus, and cytoplasmic granules. Interference-contrast microscopy, × 1,250. (b) The interaction between log-phase type 4 gonococci and human PMN leukocytes incubated for 30 min in the presence of fresh human serum. The leukocyte is rounded up and two large phagocytic vacuoles containing organisms can be seen. Interference-contrast microscopy, × 1,250.

TABLE III

Interaction of Gonococci and Human Polymorphonuclear Leukocytes

		Leukocytes	
Gonococci	10% human serum	Attach-ment	Inges-tion
Type 1 and 2 (log phase)	Fresh	+	0
	Heat inactivated	+	0
Type 1 and 2 (stationary phase)	Fresh	+	+
	Heat inactivated	+	±
Type 4 (log phase)	Fresh	±	−
	Heat inactivated	±	0

surface membrane but remained free in the extracellular medium. *S. epidermidis* was ingested at the same rate in the presence or absence of allantoic fluid.

Discussion

Experimental studies of the immunobiology of *Neisseria gonorrhoeae* have been fettered by the lack of a suitable method for the laboratory cultivation of these organisms. The artificial media often employed for the cultivation of gonococci has recently been reviewed (11). Previous studies, however, have demonstrated alteration in the structural and biological properties of the gonococcus after laboratory cultivation in artificial media. These changes include loss of polysaccharide (K) antigen and resistance to the cidal effect of fresh serum (10, 16). Due to these experimental difficulties associated with the cultivation of gonococci in artificial media, other techniques have recently been reported. These culture methods include complex media supplemented with human prostate extracts (17), tissue culture (18), and endothelialized polyethylene spherules in the subcutaneous tissue of experimental animals (19). Attempts in this laboratory to employ these systems of cultivation have been uniformly unsuccessful. Growth of gonococci was relatively poor and inconsistent amongst the strains tested. Furthermore, reversion of virulent to avirulent types was commonly observed.

The chick embryo has been used widely as an experimental animal for a variety of microbes and for the cultivation of viral and rickettsial agents (20). Gonococcal infection has been produced in the chick embryo by directly inoculating organisms on the outer surface of the chorioallantoic membrane (21). An intense localized inflammatory response was observed consistent with that seen in gonococcal urethritis. These studies have recently been confirmed employing cloned type 1 or 2 and type 3 or 4 gonococci (22). Infectivity was observed with the former but not the latter colonial types. In 1963 Walsh and co-workers reported the passage of gonococci through the allantoic cavity of 8-day chick embryos (23). Based on subsequent urethral inoculation of human male volunteers, virulence of a fresh gonococcal isolate was maintained after repeated passage through chick embryos. Virulence which had been lost after repeated subcultures on agar was restored after chick embryo passage suggesting that allantoic fluid served as a selective medium by suppressing the growth of nonvirulent organisms. Cloned colonial types were not employed in these studies. The studies reported herein also demonstrate the applicability of the allantoic cavity of the chick embryo for the laboratory cultivation of gonococci. This method is relatively simple, inexpensive, and highly reproducible. Direct inoculation of cloned colonial types and cultivation for 24 h does not appear to have significant deleterious effects on the viability of 10-day chick embryos. In addition, microscopic examination of the chorioallantoic membrane failed to demonstrate bacteria or inflammatory cells within the membrane. This finding is in contrast to the previous observations of a local inflammatory response after direct inoculation of virulent gonococci on the outer surface of the chorioallantoic membrane.

Cloned colonial types of fresh gonococcal isolates from patients with disseminated infections were employed in these studies. All colonial types were

inoculated into the allantoic cavity of embryos after a limited number of subcultures on agar. Maximal growth in the allantoic cavity was not observed until organisms were successively passed through embryos. Thus, all isolates were cultivated daily for at least 10 days in embryos before phagocytosis experiments. The generation time of log-phase gonococci in embryos placed on a reciprocating shaker was 72 min. A stationary phase of growth was observed for at least 10–16 h. Similar results could not be reproduced when embryos remained stationary or if organisms were cultivated in allantoic fluid removed from the chick embryo. The reason for this discrepancy in the patterns of growth in allantoic fluid remains unexplained at the present time. All gonococcal types, previously cloned from agar cultures, maintained their colonial characteristics throughout repeated passage in the allantoic cavity. Reversion from virulent to avirulent types which commonly occurs in broth cultures was not observed.

Previous studies of gonococci-leukocyte interactions have employed organisms washed from the surface of agar or grown in broth or in biphasic media (4–6). Because of the cidal effect of fresh serum on these organisms, heat-inactivated serum or plasma or no serum were employed in test systems. Cultivation of gonococci in the allantoic cavity of 10-day chick embryos provided a population of organisms in the same phase of growth. Log-phase gonococci resisted killing by 10% fresh normal human serum whereas many strains in the stationary phase were killed by this serum concentration. Since agar cultures contain gonococci in various growth phases, these findings may explain in part the susceptibility of these organisms to fresh human serum. In addition, whereas the six log-phase gonococcal strains used in this study remained viable in 10% fresh normal human serum, only two survived the same concentration of fresh guinea pig serum and all log-phase strains were killed by 10% fresh rabbit serum. This species difference must be taken into account in studies of gonococci-leukocyte interactions in the presence of these sera.

Previous studies of phagocytosis of gonococci have also utilized stained smears (4–6). Because of leukocyte retraction and variable staining of bacteria, quantitation by this morphologic technique may be difficult. Furthermore, the viability of test organisms cannot be assessed. Cultivation of organisms in the allantoic cavity allowed for the study of gonococci-leukocyte interactions by a modification of the Maaloe technique. These studies demonstrated that log-phase type 1 and 2 gonococci resist phagocytosis by human PMN leukocytes in the presence or absence of serum indicating that these bacteria possess antiphagocytic surface components. Recent studies suggest that gonococcal pili may have antiphagocytic properties (24). Investigations are currently in progress to confirm these findings. Demonstration of the antiphagocytic properties of a surface antigen requires examination of log-phase gonococci since phagocytosis of stationary-phase type 1 and 2 organisms occurs in the presence or absence of normal human serum. Log-phase type 4 gonococci were ingested and rapidly killed only in the presence of fresh human serum. Heat-labile serum factors are also necessary for the ingestion of other log-phase bacteria by phagocytic leukocytes (8, 25).

Previous reports have stated that gonococci resist intracellular digestion and that this property determines in part the pathogenicity of this microbe (26, 27).

Recent studies have suggested that these organisms are not killed as efficiently by human PMN leukocytes as by animal leukocytes (5, 14, 28). Although comparative studies of leukocytes from different species were not performed in this investigation, our findings demonstrate that after ingestion, gonococci do not survive nor multiply intracellularly but are rapidly killed by human PMN leukocytes. Similar findings have been reported after phagocytosis of *Neisseria meningitidis* by human PMN leukocytes (8). Furthermore, no difference in the rate of intracellular killing of meningococci by human and rabbit PMN leukocytes was detected in these studies (7, 8).

Gonococci attach to the surface membrane of human PMN leukocytes. Attachment to human macrophages has also recently been observed. Attachment appears to be a unique property of *Neisseria gonorrhoeae* since other *Neisseria* species including *N. meningitidis* remain free in the extracellular medium. These observations may explain in part the close association of gonococci with PMN leukocytes in clinical specimens. Previous reports have described the attachment of group A streptococci, viridans streptococcus, *Vibrio cholerae*, and *Myco-plasma* species to various epithelial cells (29–32). Electron microscopic studies have demonstrated that gonococci attach to urethral mucosal cells and to am-niotic cells in vitro and that attachment to the latter cell type may be mediated by surface pili (33, 34). The role of pili in the attachment of gonococci to human phagocytic leukocytes is currently being investigated.

Phagocytosis is a complex biologic process which involves the attachment of microorganisms to the leukocyte surface membrane, interiorization of the microbe, and subsequent leukocyte degranulation with discharge of lysosomal contents into the phagosome. The nonimmunologic and immunologic parameters associated with attachment and ingestion of two membrane-bound particles, glutaraldehyde-treated sheep red cells and *Mycoplasma pulmonis* have been described previously (35, 36). The phagocytic cell employed in these reports was the mouse peritoneal macrophage. More recent investigations suggest that C3 promotes particle attachment and IgG particle ingestion (37). Studies of complement and IgG receptor sites on human PMN leukocytes as well as other parameters associated with the attachment and ingestion of log-phase viable gonococci are in progress.

Summary

Cultivation of *Neisseria gonorrhoeae* in the allantoic cavity of 10-day chick embryos ensured the following necessary properties for subsequent quantitative in vitro phagocytosis studies of viable gonococci: log phase of growth, resistance to the cidal effect of fresh human serum, maintenance of colonial type, and absence of clumping. Employing a modification of the Maaloe technique, phagocytosis of log-phase type 1 and 2 gonococci by human PMN leukocytes did not occur in the presence or absence of serum. These findings indicate that log-phase type 1 and 2 gonococci possess antiphagocytic surface factors Stationary-phase organisms of the same colonial type were ingested and rapidly killed by human PMN leukocytes under similar experimental conditions, thus emphasizing the necessity to employ log-phase gonococci in the study of

phagocytosis and antiphagocytic surface factors. Log-phase type 4 gonococci were ingested and rapidly killed by human PMN leukocytes in the presence of fresh human serum but not heat-inactivated serum or in the absence of serum.

Morphologic studies demonstrated that log-phase viable gonococci attach to the surface membrane of human PMN leukocytes. Interiorization of avirulent but not virulent organisms was observed in the presence of fresh human serum. Gonococci-human PMN leukocyte interactions thus provide a model for the investigation of the nonimmunologic and immunologic parameters associated with the attachment and ingestion stages of phagocytosis.

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