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# Role of Pili in the Virulence of *Neisseria gonorrhoeae*

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Gonococci of the colonial types that are associated with virulence, types 1 and 2, have pili that enable the bacteria both to attach in vitro to human epithelial cells and to resist phagocytosis by polymorphonuclear leukocytes. These piliated gonococci also agglutinate various mammalian and chicken erythrocytes. Gonococci of an avirulent colonial type, i.e., type 4, have no pili and neither attach to epithelial cells or erythrocytes nor resist phagocytosis. Like the type 4 bacteria, mechanically or enzymatically (trypsin) depiliated type 1 gonococci failed to attach to epithelial cells and erythrocytes and were susceptible to phagocytosis. Pili of types 1 and 2 gonococci were antigenically similar. Both type 1 gonococci and pili isolated from them induced in rabbits antibody that (i) precipitated gonococcal pili in immunodiffusion, (ii) reacted with piliated gonococci as tested by indirect immunofluorescent analysis, (iii) inhibited attachment of piliated gonococci to both human epithelial cells and erythrocytes, and (iv) opsonized piliated gonococci.

Although *Neisseria gonorrhoeae* has been recognized as the cause of gonorrhea for nearly a century, little is known of the specific components of gonococci that enable them to interact with human tissues and to evade the host's antibacterial defenses. The virulence of gonococci is associated with specific colonial morphology on suitable medium and under standard growth conditions (9). The genetically determined colonial types are designated as types 1 through 4. Type 1 colonies are small (about 0.5-mm diameter), convex, round, with an entire edge, translucent, colored dark gold, and slightly viscid. Type 2 colonies are similar to those of type 1, but are friable, slightly crenated, and with a sharper edge. Type 3 colonies are larger (about 1-mm diameter), flat-edged, low convex, granular, colored light brown, and viscid. Type 4 colonies are similar to type 3, but are amorphous and colorless. Whereas gonococci from colonial types 1 and 2 caused gonorrhea in volunteers, the standard inoculum of bacteria of types 3 and 4 failed to cause disease in volunteers (9). Other properties also vary among the colonial types, e.g., autoagglutinability (9) and competence for transformation (14). Of particular interest in regard to virulence is evidence (16) that the virulent colonial types are more resistant than the avirulent types to phagocytosis by polymorphonuclear leukocytes (PMN). Because both virulent and avirulent gonococci are killed within leukocytes (16, 18, 19), these observations suggest that virulence is related to an antiphagocytic component(s) of the bacteria. Furthermore, the resistance to phagocytosis is independent of leukotoxic and metabolic activity of the bacteria (16), thus suggesting that a structural component(s) is responsible. A striking structural difference between the virulent and the avirulent colonial types of gonococci is the presence on the former, i.e., colonial types 1 and 2, of surface appendages with the appearance of pili and their absence on the latter, i.e., colonial types 3 and 4 (8, 15). The present studies were conducted to evaluate the role of pili in the resistance of the virulent colonial types of gonococci to phagocytosis by PMN. Furthermore, because pili of other bacteria are involved in "stickiness" (1-4, 17), the role of pili in promoting adhesion of the virulent colonial types of gonococci to human epithelial cells and to erythrocytes was examined.

## MATERIALS AND METHODS

**Media.** The solid medium (GCBS) was G C medium base (Difco, Detroit, Mich.) supplemented with 1% V-C-N Inhibitor (BioQuest, Cockeysville, Md.), 1% IsoVitaleX (BioQuest), and 10% of a defined supplement (9). Supplements were added to fluid

base at 45 C just before dispensing to plates. Before use, the solidified medium was incubated at 36 C for 2 or 3 days to reduce surface moisture.

The liquid medium (LGCBS) was similar except starch and agar were omitted.

**Reagents.** Chloramphenicol (Carlo Erba, Milano, Italy), mitomycin C (Sigma Chemical Co., St. Louis, Mo.), nalidixic acid (kindly supplied by Sterling-Winthrop Research Institute, Rensselaer, N. Y.), and actinomycin D (Merck, Sharp, and Dohme, West Point, Pa.) were added to sterile LGCBS immediately before use.

D-glucose and maltose (May and Baker, Ltd., Dagenham, England); D-mannitol (Fisher Scientific Co., Fair Lawn, New Jersey); and dulcitol, D-sorbitol, raffinose, saccharose, lactose, D-fructose, D-galactose, D-mannose, and inulin (Difco) were bacteriologically and chemically "pure."

Trypsin (Sigma) was dissolved in saline and neuraminidase (*Vibrio cholera* receptor-destroying enzyme; Behringwerke AG, Marburg-Lahn, Germany) was dissolved in phosphate-buffered saline with 0.01% calcium chloride immediately before use.

**Rabbits.** Adult rabbits of both sexes were obtained from local vendors by and housed in the Animal Care Center, Faculty of Science.

**Bacteria.** D. S. Kellogg, Jr., Center for Disease Control, Atlanta, Ga., provided *N. gonorrhoeae* strain F62 colonial types 1, 2, and 4. Cultures were maintained by serial subculture and were grown for 16 to 20 h in a candle jar at 36 C. Specific colonial types were used as inocula.

After growth, gonococci were collected as described before (16). Briefly, the growth on plates on which the desired colonial type predominated (>99% of the total colonies) was disaggregated with a glass rod and washed from the surface with warm (37 C) LGCBS. After agitation for 3 min on a Vortex mixer (Scientific Industries, Inc., Springfield, Mass.), the bacteria were concentrated by centrifugation at 1,400 × *g* at 26 C for 20 min and resuspended in LGCBS or 0.85% NaCl (saline). Viability usually exceeded 90%. Suspensions were discarded if significant clumping occurred.

Gonococci were enumerated by direct microscope count with a Petroff-Hausser chamber (C. A. Hausser and Son, Philadelphia, Pa.).

Gonococcal suspensions of about $10^8$ cells per ml were blended (1) at 4 C for 2 min at top speed in an Omnimixer (Ivan Sorvall, Inc., Norwalk, Conn.). After blending, the bacteria were collected by centrifugation as above.

Gonococci were incubated at 37 C for 30 min in trypsin (1,000 μg/ml) dissolved in saline. The bacteria were washed twice with saline, collected by centrifugation at 1,400 × *g* at 26 C for 20 min, and suspended in medium.

For experiments involving incubation after modification, the blended or trypsinized bacteria were resuspended in LGCBS or in LGCBS containing a drug, incubated in a candle jar at 36 C for 90 min, and collected by centrifugation as above.

Gonococci suspended in saline were disrupted in a sonic oscillator (Raytheon Co., Waltham, Mass.) at 10 kc at 0 C for 5 min.

**Pili.** Pili were isolated and purified by an adaptation of Brinton's procedure (1). Gonococci were suspended in saline and blended. After centrifugation at 1,400 × *g* at 26 C for 20 min, the sediment was discarded, and the supernatant fluid was adjusted to pH 3.9 with 1 N HCl and held overnight at 4 C. The aggregate was collected after centrifugation at 2,000 × *g* at 4 C for 30 min and dispersed in saline. The pH was adjusted to 7 with 0.5 N NaOH. After addition of $MgCl_2$ to a final concentration of 0.1 M, the aggregates that formed were collected by centrifugation as above. The sedimented pili were dispersed in saline, thrice resedimented by $MgCl_2$ and centrifugation, and finally dispersed in saline and stored at 4 C. The protein concentration, as determined by the biuret method (20), was 0.95 mg/ml. The purified pili gave a single precipitin band with a reaction of identity in immunodiffusion (below) with rabbit antisera (below) to type 1 gonococci and to isolated pili from type 1 gonococci (Fig. 1). Injection of rabbits with the purified pili from type 1 gonococci stimulated antiserum that gave a single precipitin band with a reaction of identity in immunodiffusion with pili from type 1 gonococci and with sonically disrupted type 1 gonococci, but no visible precipitate with sonically disrupted type 4 gonococci (Fig. 1).

**Antisera.** Before and 1 week after a course of immunization, blood was obtained from rabbits and allowed to clot at 4 C. After centrifugation at 4 C, serum was collected, divided, and stored at −65 C. The initial injection of a course of immunization with gonococci was given into the footpads and was 1 ml of 50% (vol/vol) bacteria (about $10^{9.7}$ cells in saline) and incomplete Freund adjuvant (Difco). On days 2 and 4 thereafter, 1 ml of the same suspension was injected subcutaneously. On days 6, 8, and 10, 1 ml, 1.5 ml, and 2 ml, respectively, of a saline suspension of about $10^{9.7}$ bacteria per ml were injected intramuscularly. The course of immunization with purified pili of type 1 gonococci (above; 0.95 mg of protein/ml) was similar.

For each antiserum, comparison to preimmunization serum by a variety of immunological reactions, including the indirect fluorescent antibody technique (IFAT; below) and immunodiffusion (below) against sonically disrupted bacteria, indicated that antibody was formed that reacted with the bacterium or the component that was used for immunization, e.g., antiserum to type 4 gave a precipitin reaction in immunodiffusion with sonicated type 4 bacteria (Fig. 1).

**Immunodiffusion.** Immunodiffusion (12) was carried out in 1% special Noble agar (Difco) in saline. After the precipitin bands had developed fully and been examined, the gel was stained to seek additional bands. The gel was washed, first with saline and then with water, and allowed to dry. Precipitin bands were stained with 0.6% amido schwartz 10B (E. Merck AG, Darmstadt, Germany) in methanol-acetic acid-water (45:10:45).

**IFAT.** Acetone-fixed smears of gonococci or of mammalian cells were incubated with a 1:10 dilution of test rabbit antiserum (or preimmunization control serum) at 37 C for 30 min, washed with phosphate-buffered saline (pH 7.2) for 10 min, and reacted at 37 C for 30 min with sheep antiserum to rabbit globulin

conjugated with fluorescein isothiocyanate (Miles Laboratories, Inc., Kankakee, Ill.). The slides were examined with a Leitz fluorescent microscope.

**Absorption of sera.** Bacteria were suspended in saline to a concentration of $10^{10}$ cells/ml, and pili were used in a concentration of 0.95 mg of protein/ml. A volume equal to that of the serum to be absorbed was centrifuged at 1,400 × *g* for 30 min at 4 C for bacteria and 2,000 × *g* for 30 min at 4 C for pili. The supernatant fluid was discarded, and a 1:2 dilution of serum in saline was mixed with the bacteria or the pili and incubated, first at 37 C for 30 min and then at 4 C for 16 h. The serum was harvested after centrifugation as above. The absorbed sera were negative for bacteria on smear and for residual pili on testing by immunodiffusion against unabsorbed rabbit antipilus serum.

**Electron microscopy.** Gonococci or purified pili suspended in saline were mixed with an equal volume of 1% sodium phosphotungstate, pH 6.8. The mixture was placed on collodion-coated grids, allowed to dry, and examined in a Hitachi S-8 electron microscope. Sukhum Bunyaratvej, Department of Pathology, Ramathibodi Faculty of Medicine, Mahidol University, expertly advised on and assisted with the electron microscopy.

**Phagocytosis.** PMN-rich leukocytes were collected in modified Hanks solution (10) that contained 0.01% bovine serum albumin (Armour Pharmaceutical Co., Chicago, Ill.) and 0.1% glucose (HBG) from sterile peritoneal exudates induced in rabbits by the intraperitoneal injection, 24 h earlier, of 250 ml of 1% shellfish glycogen (Mann Research Labs, New York, N.Y.). Human leukocytes were collected from donors who denied a history of gonococcal infection. The leukocyte-rich fraction was aspirated from peripheral blood after sedimentation at 26 C for 90 min in 25% (vol/vol) of 6% dextran (Pharmacia, Uppsala, Sweden) and 10 U of heparin (Normark-werke, Hamburg, Germany) per ml. PMN were tested for phagocytosis of gonococci in the "dilute" phagocytic test system (13, 16). Briefly, 1.25 × $10^8$ leukocytes were concentrated in a screw-capped tube (13 by 100 mm) by centrifugation at 230 × *g* at 4 C for 5 min and mixed with 6.25 × $10^8$ gonococci contained in a volume of 0.025 ml and with 0.5 ml of appropriate diluent, e.g., HBG or serum. The mixture was tumbled end-over-end at 12 rpm at 37 C for 30 min. After incubation, smears were prepared, stained with Loeffler's alkaline methylene blue, and examined with a microscope. Results were expressed as the percentage of 400 PMN that contained at least 1 gonococcus. The test was reproducible, i.e., the mean difference between duplicates was <10% of the mean percentage of phagocytosis. The 95% confidence level was used for the significance of differences.

**HA.** Blood was collected in an equal volume of 0.06 M sodium citrate. The erythrocytes were washed three times with saline and suspended in saline to 3% (vol/vol) concentration. Rabbit erythrocytes were used routinely. The hemagglutination (HA) test was a modification of that of Tweedy (17); 0.05 ml of gonococci ($10^{10}$ bacteria/ml) or of pili (0.95 mg of protein/ml), 0.05 ml of saline, and 0.1 ml of 3% erythrocytes were mixed in a tube (10 by 75 mm), sealed with plastic film, incubated at 37 C for 20 min and then at 4 C for 16 h, and examined for agglutination. To determine the effect of trypsin (1,000 μg/ml) and of neuraminidase (10%) treatment of erythrocytes on hemagglutination by type 1 gonococci, the erythrocytes were reacted with enzyme at 37 C for 30 min, recovered by centrifugation at 230 × *g* at 4 C for 10 min, and used in the HA test. The test for inhibition of HA by sugars was as in the HA procedure except 10% sugar solution was used as diluent.

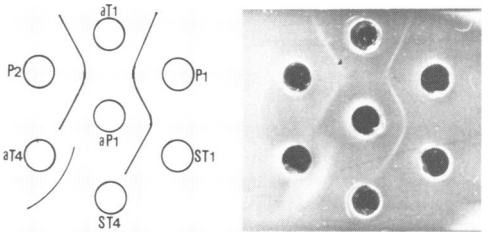


FIG. 1. *Immunoprecipitation of gonococcal antigens by antisera to piliated and nonpiliated gonococci and to isolated gonococcal pili. The drawing on the left indicates the contents of the wells and the precipitin lines of the unstained immunodiffusion reactions shown in the photograph on the right. Abbreviations: aT1 and aT4, rabbit antibody to types 1 and 4 gonococci, respectively; aP1, rabbit antibody to pili isolated from type 1 gonococci; ST1 and ST4, sonically treated types 1 and 4 gonococci, respectively; and P1 and P2, isolated pili of types 1 and 2 gonococci, respectively.*

**HAI.** The test for HA inhibition by antibody (HAI) was as the HA procedure except (i) twofold dilutions of serum in saline replaced the saline and (ii) the serum and type 1 gonococci were incubated at 37 C for 20 min before addition of erythrocytes. The HAI titer was the greatest dilution that completely inhibited HA compared to a parallel control using normal or preimmune serum.

**Hemadsorption.** Colonial growth on GCBS was flooded with 3% rabbit erythrocytes in saline (above). After 15 min at 37 C, the surface of the medium was gently washed with saline. The colonies were examined with a microscope for adherence of erythrocytes.

**Adherence to epithelial cells and PMN.** Epithelial cells were scraped (7) from the buccal mucosa of healthy humans who denied a history of gonococcal infection. The cells were concentrated by centrifugation at 230 × *g* at 4 C for 5 min, washed three times with saline, and suspended in saline to $10^5$ cells per ml. One-tenth milliliter of cells, 0.05 ml of gonococci ($10^8$ bacteria/ml), and 0.05 ml of saline were mixed in a tube (10 by 75 mm), sealed with plastic film, and incubated at 37 C for 20 min in a rotary shaker (New Brunswick Scientific Co., New Brunswick, N.J.). After incubation, a loop of suspension was examined with a microscope for adherence of gonococci to the epithelial cells. A test for epithelial adherence (EA) was considered positive if ≥80% of the epithelial cells had bacteria adhering to the surface. Typically, 20 to 40 gonococci adhered to a cell in a positive prepara-

tion. Cells with adherent bacteria often were clumped (agglutinated) by the bacteria.

Leukocytes were collected as above, incubated in 40 mM sodium fluoride dissolved in HBG at 37 C for 10 min, collected by centrifugation at 230 × *g* at 4 C for 5 min, and tested as with epithelial cells.

**EAI.** The test for inhibition of epithelial adherence (EAI) was performed as the EA procedure except (i) twofold dilutions of serum replaced the saline and (ii) the type 1 gonococci and the serum were incubated at 37 C for 20 min before addition of the buccal epithelial cells. The EAI titer was the greatest dilution that caused marked reduction (to <10% of cells with adherent gonococci) of adherence compared to a parallel control performed with normal or preimmune serum.

**Opsonization.** Twofold dilutions in HBG of preimmunization and of postimmunization sera were used as diluent in the phagocytic test. Opsonization was considered to have occurred if the immune serum yielded 50% greater phagocytosis than did the preimmune serum at the same dilution. Because undiluted or slightly diluted normal rabbit serum clumped leukocytes and, thereby, reduced phagocytosis, the least dilution of serum used in opsonic tests was 1:16.

## RESULTS

**Piliation of cultured and of experimentally modified gonococci.** Electron photomicrographs of type 1 gonococci revealed numerous hair-like surface appendages approximately 8.5 nm in diameter and of varying length (Fig. 2a). The appendages resembled pili of other bacteria and were similar in appearance to those described before (15) on whole gonococci of colonial types 1 and 2. Type 1 gonococci that were blended had no discernible pili (Fig. 2d) and resembled type 4 organisms (Fig. 2c). Trypsin-treated type 1 gonococci (not shown) were similar in appearance to the blended bacteria. Incubation of mechanically depiliated type 1 gonococci in LGCBS for 90 min resulted in reformation of pili (Fig. 2f). An inhibitor of protein synthesis at the translational level, i.e., 5 μg of chloramphenicol per ml, blocked discernible regeneration of pili by depiliated type 1 gonococci (Fig. 2f).

Figure 2b is an electron photomicrograph of pili that were isolated from type 1 gonococci. The aggregates of pili resembled those described by Swanson et al. (15). The purified preparation of pili was free of recognizable cells, other organelles, and membrane and wall fragments.

Immunological analyses were consistent with the electron microscope observations. As determined by IFAT, rabbit antibodies formed during immunization with purified type 1 pili reacted with type 1 gonococci, but with neither type 4 gonococci nor mechanically or enzymatically depiliated type 1 gonococci. The reactivity with antipilus antibody was regained during incubation of depiliated type 1 bacteria in LGCBS, and the IFAT was used to follow the formation of pili by the previously depiliated bacteria. Faint immunofluorescence was first detected in samples taken after 5 min of incubation. The fluorescence gradually increased in intensity thereafter and reached a maximum at 30 min of incubation.

**Interactions of cultured and of experimentally modified gonococci and of gonococcal pili with mammalian cells.** As reported before (16), type 1 gonococci were significantly more resistant than type 4 organisms to phagocytosis by PMN (Fig. 3). Both mechanical and enzymatic depiliation of the type 1 gonococci significantly reduced their resistance to phagocytosis (Fig. 3). Incubation of depiliated type 1 bacteria in conditions that permitted regeneration of pili (above) was associated with an increase in resistance to phagocytosis; the cells regained antiphagocytic activity equal to that of freshly grown type 1 gonococci (Fig. 3). Addition of inhibitors of protein synthesis at both the translational level, i.e., chloramphenicol (5 μg/ml), and the transcriptional level, i.e., actinomycin D (1 μg/ml) and mitomycin C (10 μg/ml), during incubation significantly ($P < 0.05$) inhibited the restoration of the antiphagocytic activity of mechanically depiliated type 1 gonococci (Fig. 3). Inhibition of deoxyribonucleic acid replication by nalidixic acid (2.5 μg/ml) during incubation of depiliated cells did not, however, inhibit the reacquisition of the antiphagocytic activity; after incubation with this compound, mechanically depiliated gonococci were as resistant to phagocytosis as freshly grown type 1 bacteria (Fig. 3).

Although rabbit PMN were used routinely for determination of the resistance of gonococci to phagocytosis, the results were typical of those with human PMN, e.g., in a representative experiment with human PMN, the phagocytic results were 10% with type 1 gonococci, 49% with type 4 gonococci, 33% with blended type 1 gonococci, and 10% with blended type I bacteria that had reformed pili.

Together these results suggested that a protein component(s) is a major determinant of the antiphagocytic properties of the virulent colonial type 1 gonococci. Furthermore, in experimentally modified gonococci the appearance and disappearance of the component(s) corresponded to the presence of pili. Because depiliation of type 1 gonococci failed to eliminate completely the antiphagocytic activity (i.e., the depiliated type 1 bacteria were somewhat more

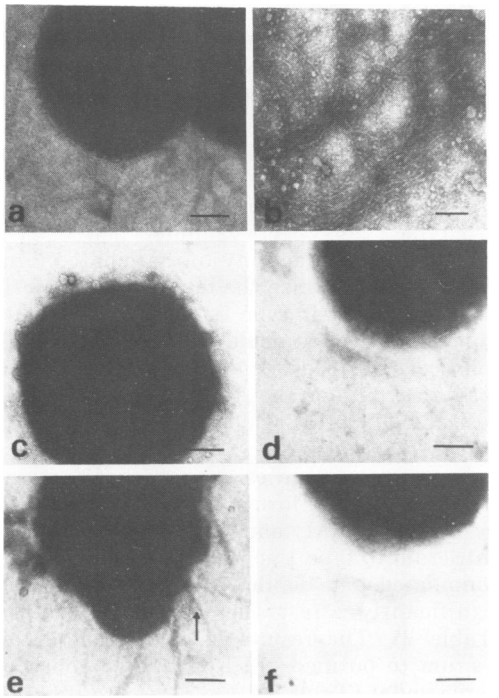


FIG. 2. *Electron photomicrographs of negatively-stained gonococci and gonococcal pili. (a and b, respectively) Type 1 gonococci with numerous pili attached to the surface and the pili isolated from type 1 gonococci; the isolated pili are aggregated. (c) Type 4 gonococci; pili are not detected. (d) Type 1 gonococci that have been blended; the surface is smooth and apparently rendered free of pili. Incubation of blended type 1 gonococci for 90 min resulted in reappearance of pili (arrow) as in (e). Chloramphenicol (5 μg/ml) during incubation of blended type 1 gonococci inhibited reformation of pili (f). (b) ×95,000; and the remainder are ×45,000. Bars represent 0.1 μm.*

resistant to phagocytosis than the avirulent colonial type 4 bacteria [Fig. 3]), the type 1 gonococci may have an additional anti-phagocytic determinant(s) that resists trypsin and shearing force.

Other reactions with mammalian cells were similar to resistance to phagocytosis in their dependence on a protein component(s) and in their correlation with the presence or absence of pili. Piliated type 1 gonococci, but neither type 4 nor depiliated type 1 gonococci, agglutinated rabbit erythrocytes (Fig. 3 and 4a). The HA resulted from multiple attachment of gonococci to erythrocytes with formation of aggregates (Fig. 4b).

Neither the 11 sugars that were tested (D-glucose, maltose, D-mannitol, dulcitol, D-sorbitol, raffinose, saccharose, lactose, D-fructose, D-galactose, D-mannose, and inulin) nor the treatment of erythrocytes with trypsin and with neuraminidase inhibited HA by type 1 gonococci.

HA was not unique to rabbit erythrocytes; type 1 gonococci also agglutinated other mammalian erythrocytes, i.e., guinea pig, sheep, and human type O, $Rh^+$ erythrocytes, and those of one nonmammalian species that was tested (chicken).

The attatchment of erythrocytes to type 1 gonococci was also manifested by hemadsorption on colonies (Fig. 4c). Type 4 colonies did not hemadsorb (Fig. 4d).

The reformation of pili on depiliated type 1 gonococci was associated with restoration of HA and EA activity of the bacteria (Fig. 3). Whereas 5 min of incubation resulted in immunological evidence of reformation of pili (above), HA activity was not restored until 15 min of incubation.

Piliated type 1 gonococci, but not type 4 and mechanically depiliated type 1 gonococci, adhered (i) to buccal epithelial cells (Fig. 3 and 5) and (ii) to both rabbit and human PMN in conditions in which phagocytosis was blocked by sodium fluoride.

Purified pili of type 1 gonococci also agglutinated rabbit erythrocytes with a reaction that was grossly indistinguishable from that

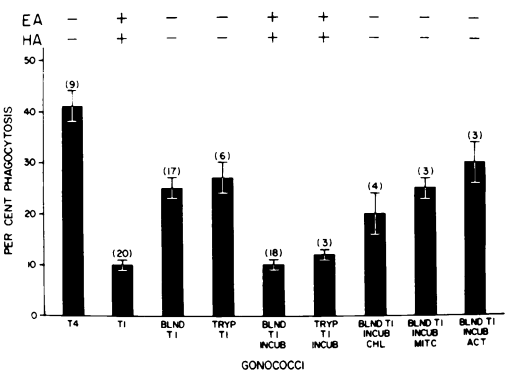


FIG. 3. *Epithelial cell adherence (EA), hemagglutination (HA), and susceptibility to phagocytosis by rabbit leukocytes of cultured and of experimentally-modified gonococci. Bars and lines represent the means, ±2 standard errors, of the number of tests indicated in parentheses. EA and HA were scored as positive (+) or negative (−). Gonococci were freshly cultured types 1 (T1) and 4 (T4) organisms. Modifications included blending (BLND), trypsin treatment (TRYP), incubation for 90 min after blending (INCUB) in medium and in medium containing 5 μg of chloramphenicol (CHL) per ml, 10 μg of mitomycin C (MIT C) per ml, or 1 μg of actinomycin D (ACT) per ml.*

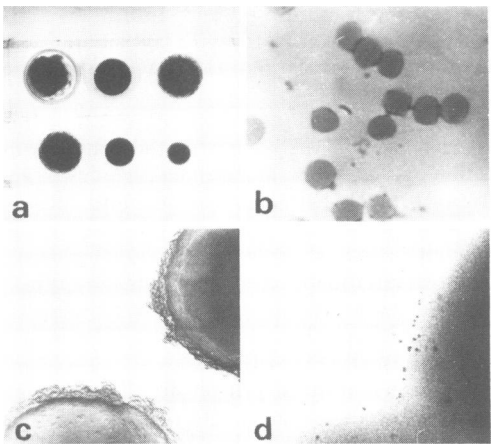


FIG. 4. *Hemagglutination by gonococci and gonococcal pili and hemadsorption to colonies of gonococci. Rabbit erythrocytes were used. (a) From left to right across the top are: type 1 gonococci, blended type 1 gonococci, and blended and incubated type 1 gonococci; and across the bottom are: isolated pili of type 1 gonococci, type 4 gonococci, and erythrocytes alone. (b) Photomicrograph of a Wright stained smear of erythrocytes and type 1 gonococci (×100). (c and d) Type 1 and 4 colonies, respectively; numerous erythrocytes are adherent to the former (×10).*

produced by whole type 1 bacteria (Fig. 4a). Adherence of purified pili to buccal epithelial cells was demonstrated by IFAT with rabbit antipilus serum (Fig. 5D). Detection of adherence of purified pili to PMN was complicated technically by the autofluorescence of the PMN. Because the autofluorescence was of a blue color, it could be differentiated from the apple-green fluorescence of the labeled antibody. By this means, attachment of purified pili to human and to rabbit PMN was detected by IFAT with rabbit antipilus serum.

The attachment of pili to PMN did not inhibit the phagocytic activity of rabbit PMN. After incubation of leukocytes with purified pili ($10^8$ PMN per 0.475 mg of protein), the PMN were equal to freshly obtained PMN in their ability to phagocytose nonpiliated type 4 gonococci (38 and 40% phagocytosis, respectively).

**Antigenicity of cultured and of experimentally modified gonococci and of gonococcal pili.** Antisera raised in rabbits were tested for opsonization of and, as possible, for HAI and EAI against homologous and other gonococci (Table 1). Piliated gonococci and pili isolated from type 1 gonococci induced antibody that (i) inhibited both HA and EA by piliated gonococci, and (ii) opsonized the inducing organisms and the other piliated gonococci. The titers of each activity of each antiserum were similar regardless of the piliated gonococci that were used in the test. Neither type 4, depiliated type 1, nor incubated but inhibited depiliated type 1 gonococci were opsonized by antisera formed against the piliated cells. Furthermore, antisera to nonpiliated gonococci (i.e., type 4, blended type 1, and blended and incubated but inhibited type 1 gonococci) exhibited neither HAI, EAI, nor opsonin activity to the piliated forms and did not opsonize the nonpiliated bacteria. That antibodies to type 1 gonococci and to pili from them reacted equally with types 1 and 2 gonococci was consistent with earlier evidence that the pili of the two types are similar antigenically (T.M. Buchanan et al., J. Clin. Invest. **72**:17a, 1972).

Absorption studies were confirmatory. Whereas piliated forms and isolated pili adsorbed HAI, EAI, and opsonic activity from antiserum to type 1 gonococci, absorption with nonpiliated and depiliated organisms did not significantly affect the antibody activities (Table 2). The results of absorption of antiserum to purified pili from type 1 gonococci were similar (Table 3).

Together, these results further suggested that pili were responsible for the adherence of virulent gonococci to epithelial cells and for their resistance to phagocytosis because antibody

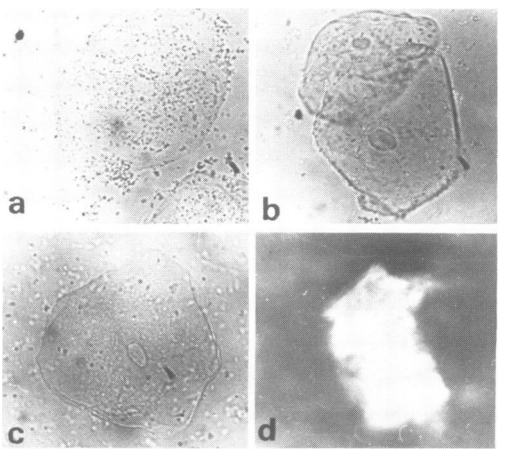


FIG. 5. *Adherence of gonococci and gonococcal pili to epithelial cells. Type 1 (a) but neither blended type 1 (b) nor type 4 (c) gonococci adhered to the surface of human buccal epithelial cells (unstained; ×40). Isolated pili attached to epithelial cells as detected by IFAT with rabbit antipilus serum (d; ×40); control preparations (not shown) of epithelial cells but not pili did not fluoresce after reaction with the antisera and presented as a dark microscope field.*

TABLE 1. *Reactions of antigonococcal and of antipilus sera with homologous and heterologous gonococci*[a]

| Antiserum to | Titer$^{-1}$ with test gonococci | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Type 1 | | | Blended type 1 | Blended type 1 incubated | | | Blended type 1 incubated, inhibited[b] | Type 4 | Type 2 | | |
| | HAI | EAI | Ops | Ops | HAI | EAI | Ops | Ops | Ops | HAI | EAI | OPs |
| Type 1 | 128 | 128 | 256 | <16 | 128 | 128 | 256 | <16 | <16 | 256 | 128 | 128 |
| Type 4 | <2 | <2 | <16 | <16 | <2 | <2 | <16 | <16 | <16 | ND | ND | ND |
| Blended type 1 | <2 | <2 | <16 | <16 | <2 | <2 | <16 | <16 | <16 | ND | ND | ND |
| Blended type 1 incubated | 64 | 64 | 64 | <16 | 64 | 64 | 64 | <16 | <16 | 128 | 128 | 128 |
| Blended type 1 incubated, inhibited[b] | <2 | <2 | <16 | <16 | <2 | <2 | <16 | <16 | <16 | ND | ND | ND |
| Type 1 pili | 128 | 128 | 64 | <16 | 128 | 64 | 128 | <16 | <16 | 128 | 64 | 64 |

[a] HAI, Hemagglutination inhibition; EAI, epithelial adherence inhibition; Ops, opsonization; ND, not done.
[b] Chloramphenicol (5 μg/ml) in medium during incubation.

TABLE 2. *Absorption of antiserum to type 1 gonococci with cultured and experimentally modified gonococci and with pili*[a]

| Antiserum to type 1 absorbed with | Titer$^{-1}$ with type 1 gonococci | | |
|---|---|---|---|
| | HAI | EAI | Ops |
| Type 1 | <2 | <2 | <16 |
| Blended type 1 | 64 | 256 | 256 |
| Blended type 1 incubated | <2 | <2 | <16 |
| Type 4 | 128 | 256 | 256 |
| Type 2 | <2 | <2 | <16 |
| Type 1 pill | <2 | <2 | <16 |
| Unabsorbed | 128 | 128 | 256 |

[a] HAI, Hemagglutination inhibition; EAI, epithelial adherence inhibition; Ops, opsonization.

TABLE 3. *Absorption of antiserum to pili of type 1 gonococci with cultured and experimentally modified gonococci and with pili*[a]

| Antiserum to type 1 pili absorbed with | Titer$^{-1}$ with type 1 gonococci | | |
|---|---|---|---|
| | HAI | EAI | Ops |
| Type 1 | <2 | <2 | <16 |
| Blended type 1 | 64 | 128 | 64 |
| Type 4 | 128 | 64 | 128 |
| Type 2 | <2 | <2 | <16 |
| Type 1 pili | <2 | <2 | <16 |
| Unabsorbed | 128 | 128 | 64 |

[a] HAI, Hemagglutination inhibition; EAI, epithelial adherence inhibition; Ops, opsonization.

that was directed against pili reversed these activities.

## DISCUSSION

To invade mucosal surfaces and tissues successfully, a bacterium must both adhere to the surface and evade the host's defenses in the tissues. Virulent colonial types of gonococci do both. An important structural difference between the colonial types that are unsuccessful invaders and virulent cells is the pili of the virulent colonial types (8, 15). The present results indicate that the pili provide virulent gonococci with a means both of attaching to human epithelium and of evading the phagocytic defenses of the host. For the latter activity, the pili confer resistance to phagocytosis; both piliated and nonpiliated gonococci are killed within leukocytes (16, 18, 19). Although pili are associated with virulence in gonococci, nonpathogenic Neisseria (e.g., *N. catarrhalis*, *N. perflava*, and *N. subflava*) may also have pili (22). The nature and properties of the pili of the nonpathogenic species have not been determined. It is, therefore, premature to speculate on their activity in relation to that of the pili of virulent gonococci.

Perhaps it seems paradoxical that a single bacterial component both promotes their attachment to epithelial cells, to PMN, and to erythrocytes and inhibits their phagocytosis by PMN. There is precedent, however, for the M protein of group A streptococci is both antiphagocytic (6) and responsible for adherence of the streptococci to epithelial cells (5). Furthermore, the present results emphasize that direct attachment of bacteria to PMN is not essential to the phagocytic process. Although nonpiliated gonococci did not detectably adhere to PMN, the bacteria were readily ingested. Piliated

gonococci, in contrast, adhered to PMN but resisted phagocytosis.

Understanding of how gonococcal pili both promote adherence to cells and are antiphagocytic awaits comprehension of the molecular details of pili and their interactions with the surface of cells. Although isolated pili reacted with PMN, i.e., attached to the PMN surface, the pili per se did not inhibit phagocytosis by PMN. Pili of several enterobacteriaceae cause these bacteria to attach to surface receptors on erythrocytes (1-4, 17); the attachment is inhibited by D-mannose (11) and, therefore, may involve sugar moities of the erythrocyte surface. Neither D-mannose nor 10 other sugars inhibited attachment of gonococci to erythrocytes. Furthermore, reaction of the erythrocytes with trypsin and with neuraminidase did not prevent attachment of pili.

The sensitivity of the gonococcal pili to trypsin and the inhibition of pilus formation by inhibitors of protein synthesis indicated that protein is a major component of the pili as suggested before (T. M. Buchanan et al., J. Clin. Invest. **72**:17a, 1972). Although types 1 and 2 gonococci differ in colonial morphology, they have in common both virulence (9) and pili that seem to be immunologically and functionally alike (T. M. Buchanan et al., J. Clin. Invest. **72**:17a, 1972).

Pili are immunogenic, and antipilus antibodies have been detected in convalescent human sera (T. M. Buchanan et al., J. Clin. Invest. **72**:17a, 1972). Both piliated type 1 gonococci and purified pili of type 1 gonococci stimulated rabbits to form antibody that (i) precipitated purified pili in immunodiffusion, (ii) reacted with piliated gonococci in the IFAT, (iii) inhibited the adherence of piliated gonococci to both erythrocytes and epithelial cells, and (iv) opsonized piliated gonococci. These observations raise the possibility of using an antigen that seems to be directly associated with the virulence of gonococci in serological testing for evidence of gonococcal infection. Furthermore, the present observations identify an antigen (i.e., pili) that seems associated with virulence and, thus, of greater importance to the analysis of the immune response of the host to gonococci than incompletely defined antigens of uncertain function. The data also suggest two ways that the immune response might help to protect man from gonococci. (i) Antibodies directed against pili can inhibit attachment of the bacteria to mucosal cells and, hence, reduce the likelihood of successful tissue invasion (21). (ii) Antipilus antibodies of the appropriate immunoglobulin classes can opsonize gonococci on the mucosa or in the tissues and, thereby, promote their ingestion and destruction by phagocytes. That man does not readily become immune to gonococcal infection suggests that either the site of infection in nature is immunologically (afferent or efferent arcs or both) isolated or that virulent gonococci possess additional virulence determinants that require other host defense mechanisms for immunity.

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