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Pili as a Mediator of the Attachment of Gonococci To Human Erythrocytes

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Isolated pure gonococcal pili were found capable of producing direct agglutination of human erythrocytes. Four different strains of gonococci were compared, and hemagglutination was produced by isolated pili or piliated gonococci but not by nonpiliated gonococci of each strain. Pili from each of the four strains were antigenically distinguishable using antisera specific for pili to agglutinate piliated gonococci, form precipitin lines in Ouchterlony immunodiffusion, or inhibit hemagglutination caused by purified pili or piliated gonococci. However, these tests also demonstrate some shared antigenicity among pili. Shared antigens among the four pili types were quantitated at ≤2.5% by radioimmunoassay. Inhibition of hemagglutination was most marked with antiserum to the homologous pili type. Inhibition of hemagglutination by antiserum to heterologous pili suggested that shared antigens on pili from B and 2686 strains of gonococci are located near the erythrocyte attachment moiety of B strain pili and removed from the attachment moiety of 2686 strain pili. These results suggest that antigenic heterogeneity of pili will prove an important factor in any efforts to use pili as a vaccine for gonorrhea.

Several investigators have presented indirect evidence that pili are important for the attachment of gonococci to mammalian cells (6, 11, 12). In one instance, antiserum to purified gonococcal pili blocked the attachment of piliated gonococci to human sperm (6). Recently, Waitkins (13), Kornasky et al. (8), and Chan and Wiseman (3) have presented evidence that piliated gonococci readily attach to human erythrocytes. They suggested that pili might be responsible for this attachment. However, no studies with isolated purified gonococcal pili or with antiserum to such pili preparations were done to directly implicate pili as mediators of the attachment.

We report herein that isolated purified gonococcal pili alone are capable of causing direct agglutination of human erythrocytes, and that antiserum to such pili preparations blocks the direct hemagglutination caused by pili or pilliated gonococci. However, the blockage of direct hemagglutination by antiserum to purified pili is usually specific for the homologous pili type.

MATERIALS AND METHODS

Strains of *Neisseria gonorrhoea*. Four strains of gonococci were used, and all were from patients with gonococcal urethritis. Three strains were from Douglas Kellog (2686) or Robert Arko (M, B) of the Center for Disease Control, Atlanta, Ga., and the fourth strain (33) was from Kenneth Johnston, the Rockefeller University, New York (present address: Dept. of Microbiology, University of Texas, Dallas).

Purification of gonococcal pili and criteria for purity of pili preparations. Gonococcal pili were purified from type 2 gonococci as previously described (1). The purity of pili preparations was assessed by electron microscope observation and electrophoresis in 10% polyacrylamide gels containing 0.1% sodium dodecyl sulfate (SDS) as reported earlier (1, 2). Pili preparations used in these studies appeared free of contaminants when observed by electron microscopy and gave a single protein peak when assessed by SDS-polyacrylamide gel electrophoresis (Fig. 1, 2).

Preparation of antiserum to purified pili preparations. Fifty micrograms of purified pili protein emulsified in complete Freund adjuvant was injected in equal amounts intramuscularly and into the subscapular region of large New Zealand white rabbits. One to three boosters of 50 μg of pili emulsified in incomplete Freund adjuvant were given at weekly intervals beginning 3 weeks after the initial injection. When potent antiserum had developed, as tested by capillary precipitation, the rabbit was bled by intracardiac injection and the serum was separated and stored at −20 C centrigrade. All antisera used in this study were absorbed with homologous nonpiliated gonococci (colony type 4 of Kellogg [7]) to insure their specificity for pili.

Studies of antigenic similarity for pili from different strains. Purified gonococcal pili from strains

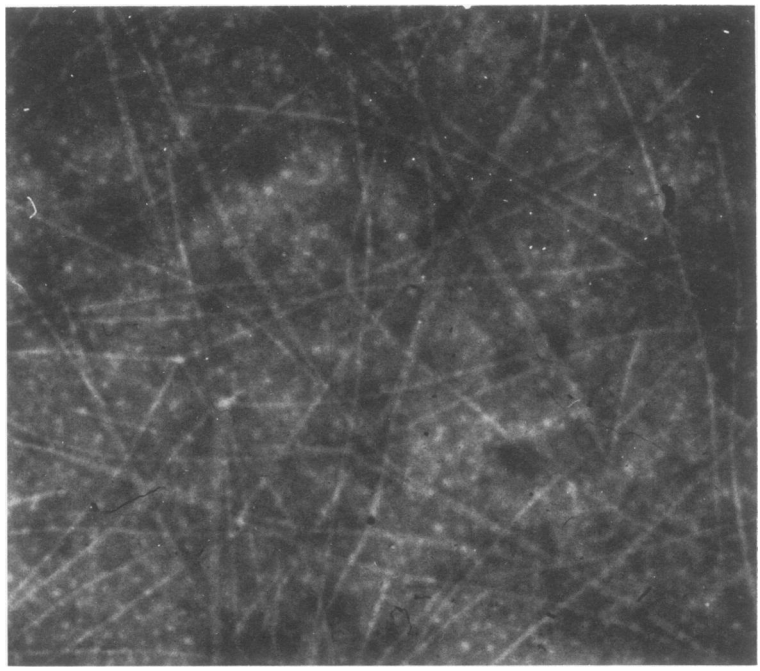

FIG. 1. *Purified gonococcal pili from strain 2686 (×125,000). Pili from strains B, 2686, 33, and M were morphologically indistinguishable.*

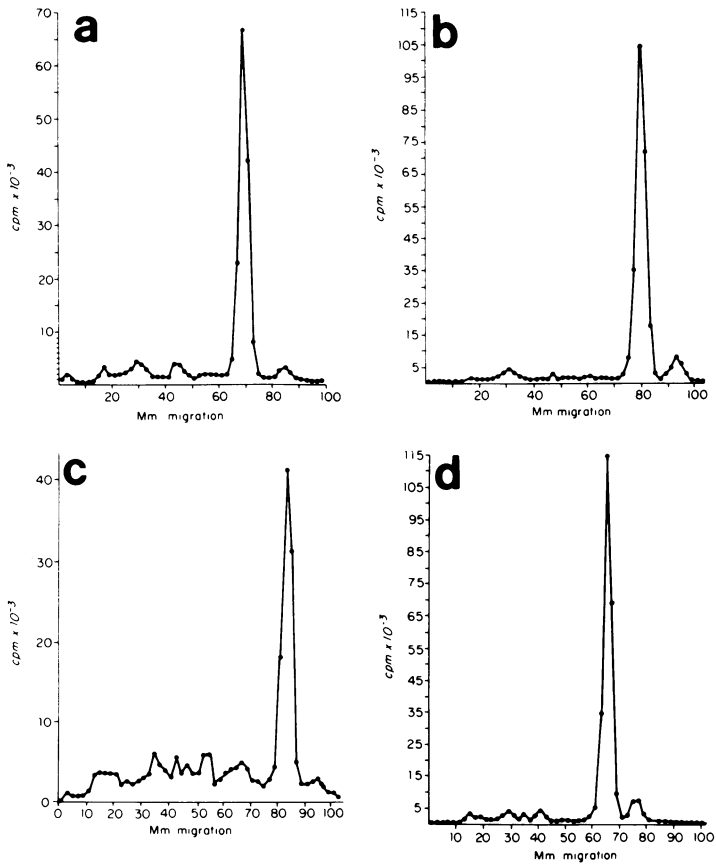

FIG. 2. *SDS-10% polyacrylamide gel pattern of ^{125}I-labeled purified pili from strains B(a), 2686(b), 33(c), and M(d).*

2686 and 33 are distinguishable antigenically by radioimmunoassay as reported previously (1), and identical radioimmunoassay procedures were used in this study for the four pili preparations. Further evidence of antigenic heterogenicity of gonococcal pili was obtained herein using direct agglutination of piliated gonococci from strains 2686, 33, M, or B by antiserum to purified gonococcal pili from each of these strains. Also purified gonococcal pili were tested in Ouchterlony double immunodiffusion (10) against antisera prepared against homologous and heterologous purified pili. Pili preparations used for immunodiffusion were adjusted to 1% bovine serum albumin and sonicated for 5 min in a bath sonicator to reduce their size sufficiently to allow diffusion through the gel. Precipitin reactions were observed daily and allowed to develop for 96 h. Gels were then washed in normal saline at 4 C for 3 days before drying and staining with 0.25% Coomassie brilliant blue (Sigma Chemical Co., St. Louis, Mo.) in methanol, acetic acid, and water (9:2:9) and destaining with ethanol, acetic acid, and water (9:2:9).

Direct hemagglutination. Blood group O or A Rh+ human blood was obtained in heparinized tubes, and the cells were separated from the serum by low-speed centrification and washed twice with phosphate-buffered saline, pH 7.3. The washed blood was used immediately or a portion was stored at 4 C for up to 2 weeks at a 40% concentration (vol/vol) in Alsever solution (5). Before use, erythrocytes were again washed in phosphate-buffered saline and resuspended to a 3% (vol/vol) concentration for use in hemagglutination studies. Type 1 or type 2 colonies (7) from a 15- to 17-h growth of gonococci were adjusted to an optical density of 0.3 at 560 nm (approximately 10^8 gonococci/ml), and equal volumes of this suspension and the 3% erythrocytes were gently mixed on a glass slide. Strong hemagglutination began appearing within 15 s, and all hemagglutination accepted for these studies occurred within 4 min of gently mixing erythrocytes and piliated gonococci. Older cultures of piliated gonococci and colony types 1 and 2 gonococci that were lightly piliated did not hemagglutinate as effectively as young cultures of heavily piliated gonococci. Colony types 3 or 4 gonococci (7) did not cause hemagglutination.

RESULTS

Figure 2 is the SDS-10% polyacrylamide gel pattern of ^{125}I-labeled purified pili from strains B, 2686, 33, and M. In each case a single major protein peak was found. The subunit molecular weights of pili from each strain varied slightly, with 2686 and B pili at approximately 20,000 daltons. Strain 33 pili were smaller and M pili were larger by 500 to 1,000 daltons. Antisera to these pili preparations after absorption with homologous T4 organisms showed no reactivity with untreated, sonicated, or deoxycholate-solubilized homologous T4 gonococci, but possessed high antibody levels to their specific strain of pili. Figure 3 demonstrates the strong agglutination of piliated gonococci produced by homologous anti-pili antiserum, and no significant agglutination caused by heterologous anti-pili antiserum.

Table 1 summarizes the results of direct agglutination of piliated gonococci by antiserum to purified pili from homologous or heterologous strains. In each instance maximal agglutination was produced by the homologous anti-pili antiserum and partial to no agglutination was caused by heterologous anti-pili antiserum. No agglutination of piliated gonococci was produced by normal rabbit serum, and none of the antisera to pili produced agglutination of nonpiliated organisms. B strain pili appeared to possess the most antigens common to other pili and M strain pili the least common antigens (Table 1).

Figure 4 summarizes the agar gel immunodiffusion results at 96 h of reaction time for purified pili reacted with homologous and heterologous pili antisera. The antiserum to B strain pili formed precipitin lines with all four strains of pili, though B strain pili reacted strongly only with its homologous antiserum.

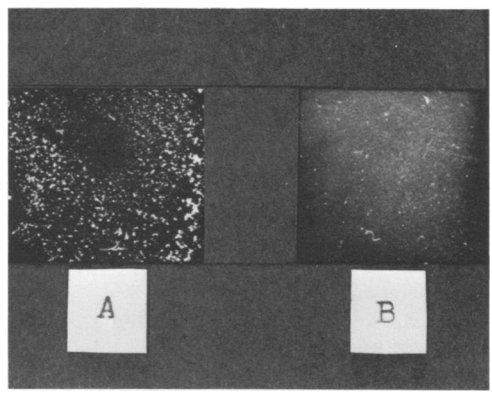

FIG. 3. *Direct agglutination of piliated gonococci strain 2686 produced by antiserum to 2686 pili (a), and no agglutination produced by reacting the same organisms with antiserum to M pili (b).*

TABLE 1. *Agglutination of piliated gonococci by antiserum to homologous or heterologous pili*

Piliated strain	Antiserum				
	B	M	2686	33	NRS[a]
B	4+	0	3+	3+	0
M	0	4+	0	0	0
2686	3+	0	4+	0	0
33	1+	0	0	4+	0
NP[b] B, M 2686, 33	0	0	0	0	0

[a] Abbreviations: NRS, Normal rabbit serum.
[b] NP, Nonpiliated.

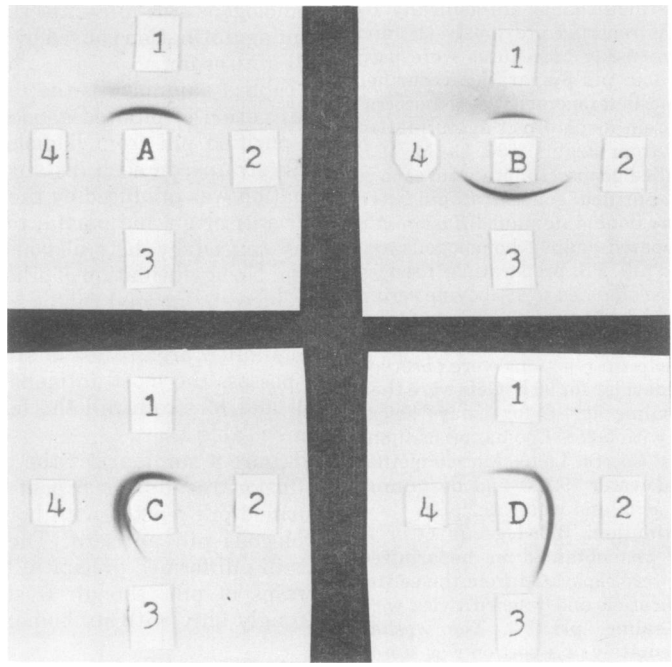

FIG. 4. *Reaction of sonicated purified gonococcal pili (central wells) with antiserum to purified pili (peripheral wells) by agar gel immunodiffusion. Pili from strains B(A), 2686(B), 33(C), or M(D). Pili antisera B(1), M(2), 2686(3), and 33(4).*

Pili from 2686, 33, and M strains each reacted only to their own antiserum and B pili antiserum (Fig. 4). Intensity of precipitin lines as well as spurring suggested in each instance of cross-reactivity that the homologous antiserum recognized some antigen(s) not identified by heterologous antisera.

To quantitate the degree of shared antigenicity among pili, the binding of ^{125}I-labeled pili from strains B, 2686, 33 and M by homologous antisera was inhibited with unlabeled pili from each strain (Fig. 5). Shared antigens generally accounted for less than 1% of the weight of each pili type. The most shared antigenicity was present for pili from strains 2686 and B, with B pili containing approximately 2.5% (50 ng/2,000 ng × 100) 2686 antigen by weight (Fig. 5d).

Figure 6 illustrates the direct agglutination of human erythrocytes by isolated gonococcal pili. This agglutination was usually somewhat weaker than that produced by piliated gonococci, and both could be blocked by antiserum to purified pili as summarized in Table 2. Homologous pili antiserum was most effective at blocking hemagglutination, though weak inhibition of hemagglutination caused by B strain gonococci was produced by 2686 and 33 pili antiserum. Similarly, B pili antiserum produced weak inhibition of hemagglutination mediated by strain 33 gonococci. In contrast, hemagglutination produced by piliated 2686 gonococci was enhanced by antiserum to B pili. This might suggest that the B and 2686 shared antigen(s) was located near the attachment region in the B pilus and away from the attachment region in the 2686 pilus. Figure 7 illustrates some of the effects of pili antiserum on hemagglutination produced by pili or piliated gonococci.

DISCUSSION

This study confirms previous reports indicating that gonococcal pili from different strains of gonococci are antigenically distinguishable (3, 5). Antigenic heterogeneity among pili has now been demonstrated by direct agglutination (Table 1), Ouchterlony immunodiffusion precipitation (Fig. 4), inhibition of direct hemagglutination (Table 2, Fig. 7), radioimmunoassay (Fig. 5), and immunoelectron microscopy (9) methods. However, other antigens are shared among many gonococal pili, as shown in these studies (Table 1, 2, Fig. 4, 6, 7), and with previous direct fluorescent antibody and radioactive antigen-binding assay techniques (2, 4). The percentage of shared antigens on the gonococcal pili in this study was quantitated at $\leq$2.5% (Fig. 5). Of further particular interest is the location of shared antigens on different pili.

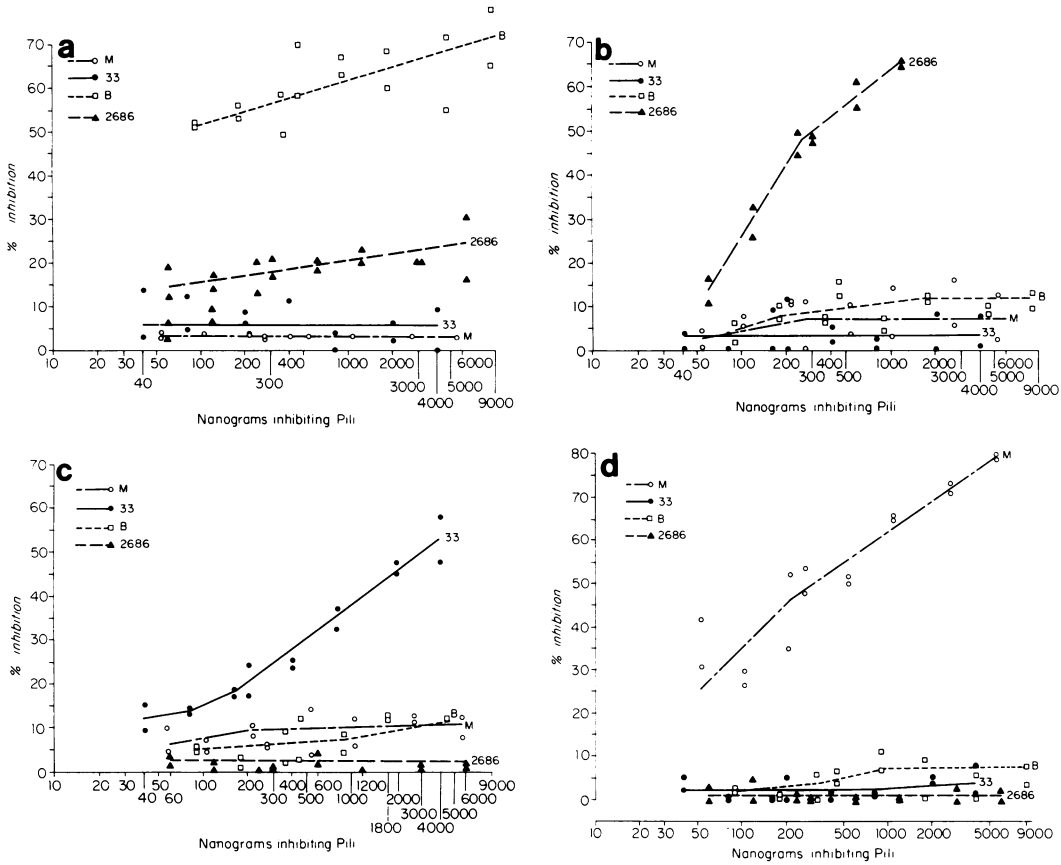

FIG. 5. *Quantitation of shared antigens among pili from gonococcal strains B, 2686, 33, and M. (a) Inhibition of binding of ^{125}I-labeled B pili by unlabeled pili B, 2686, 33, and M strain gonococci. (b) Inhibition of ^{125}I-2686 pili binding by 2686, 33, M, and B pili. (c) Inhibition of ^{125}I-33 pili binding by 33, M, B, and 2686 pili. (d) Inhibition of ^{125}I-M pili binding by M, B, 2686, and 33 pili. (Pili antiserum in each case homologous to the ^{125}I-labeled pili.)*

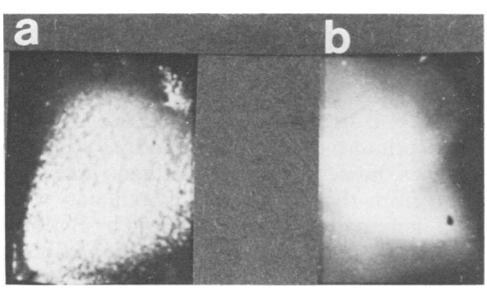

FIG. 6. *Direct agglutination of human erythrocytes by B strain pili (500 μg/ml) (A) in phosphate-buffered saline with 0.3% bovine serum albumin (pH 7.3) compared with no hemagglutination in the same buffer solution without pili (B).*

TABLE 2. *Pili-mediated hemagglutination in presence of antiserum to pili*

Piliated strain causing hemagglutination	Antiserum				
	B	M	2686	33	NRS
B	0	4+	1-2+	2+	4+
M	4+	1+	4+	4+	4+
2686	4+	4+	1+	4+	4+
33	2+	4+	4+	0	4+

These data indicate that pili are at least one mediator of the attachment of piliated gonococci to human erythrocytes, since isolated pure pili are capable of causing hemagglutination and antiserum specific for pili blocks this hemagglutination and the hemagglutination produced by piliated gonococci. This direct demonstration of the pili attachment role confirms reports by Waitkins (13), Koransky et al. (8), and Chan and Wiseman (3), who suggested pili were important for hemagglutination on the basis of indirect evidence. Assuming 100 pili

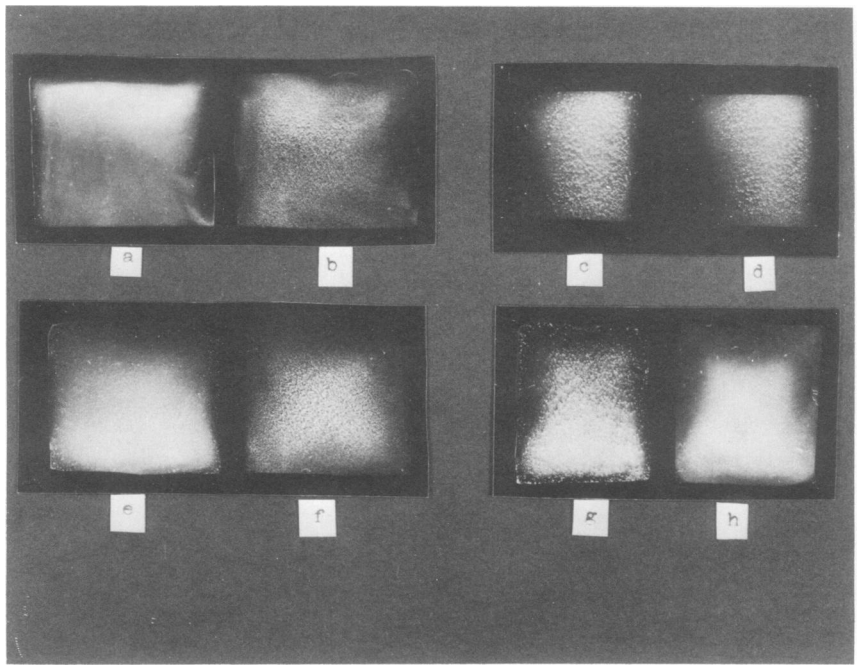

FIG. 7. *Effect of pili antiserum on pili-mediated hemagglutination. (a) Blocked hemagglutination: B gonococci, B pili antisera and human erythrocytes (HRBCs) (b) Hemagglutination: B gonococci, normal rabbit serum (NRS), HRBCs. (c) Hemagglutination: M gonococci, NRS, HRBCs. (d) Hemagglutination: M gonococci, 2686 pili antiserum, HRBCs. (e) Partially blocked hemagglutination: B gonococci, 2686 pili antiserum, HRBCs. (f) Hemagglutination: B gonococci, NRS, HRBCs. (g) Enhanced hemagglutination: 30 s after mixing 2686 gonococci, B pili antiserum, HRBCs. (h) Hemagglutination not yet developed: 30 s after mixing 2686 gonococci, NRS, HEBCs. The reaction (a) was performed simultaneously with (b), (c) with (d), (e) with (f), and (g) with (h).*

per piliated gonococcus, approximately 300- to 1,000-fold more isolated pili are required to produce hemagglutination than the number of pili present on piliated gonococci causing agglutination of a comparable number of erythrocytes. The reason for this difference has not been determined. However, several possibilities might be mentioned. This might be due to the small size of a pilus molecule relative to gonococcus, since two erythrocytes bound together by a piliated gonococcus may be further apart than those bound by a single pilus molecule. Alternatively, if one assumes that end-on attachment of pili to erythrocytes is necessary to cause hemagglutination, the favorable orientation of pili protruding from a gonococcus may markedly enhance the likelihood of attachment as compared to isolated nonoriented purified pili. A third possibility is that pili are univalent, and that only pili aligned in bundles with some attachment ends protruding from each end of the bundle could cause hemagglutination. A fourth possibility is that pili are univalent or bivalent, but that nearly all the pili in purified preparation exist as aggregated bundles. Thus, if each bundle contained 100 to 200 pili, it might perform the function of only 2 pili on a piliated gonococcus.

The hemagglutination inhibition results suggest that shared antigens of gonococcal pili may in some instances be located near the portion of the pilus involved with attachment to erythrocytes (i.e., B strain hemagglutination inhibited by 2686 pili antiserum; Table 2, Fig. 7). In other instances, however, shared antigens appear removed from the pili attachment moiety (2686 strain hemagglutination enhanced by B pili antiserum). The enhancement of hemagglutination by heterologous pili antiserum may result from bridging of piliated gonococci attached to erythrocytes. If blockage of attachment of piliated gonococci to erythrocytes by pili antiserum is representative of the attachment of piliated gonococci to epithelial cells, then pili antiserum would be expected to have minimal blocking effects on the attachment of many piliated gonococci to urethral or cervical cells. Thus, the potential value of pili as a vaccine

against gonorrhea would appear decreased by the multiple specificities of antibodies required to block pili attachment.

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LITERATURE CITED

1. **Buchanan, T. M.** 1975. Antigenic heterogenicity of gonococcal pili. J. Exp. Med. 141:1470-1475.
2. **Buchanan, T. M., J. Swanson, K. K. Holmes, S. J. Kraus, and E. C. Gotschlich.** 1973. Quantitative determination of antibody to gonococcal pili. Changes in antibody level with gonococcal infection. J. Clin. Invest. 52:2896-2909.
3. **Chan, K., and G. M. Wiseman.** 1975. A new colonial type of *Neisseria gonorrhoeae*. Br. J. Vener. Dis. 51:251-256.
4. **Eschenbach, D. A., T. M. Buchanan, H. M. Pollock, P. S. Forsyth, E. R. Alexander, J. S. Lin, S. P. Wang, B. B. Wentworth, W. M. McCormack, and K. K. Holmes.** 1975. Polymicrobial etiology of acute pelvic inflammatory disease. N. Engl. J. Med. 293:166-171.
5. **Frankel, S., S. Reitman, and A. C. Sonnenwith (ed.).** 1970. Gradwohl's clinical laboratory methods and diagnosis, vol. 2, p. 1558. C. V. Mosby Co., St. Louis, Mo.
6. **James-Holmquest, A. N., J. Swanson, T. M. Buchanan, W. D. Wende, and R. P. Williams.** 1974. Differential attachment by piliated and non-piliated *Neisseria gonorrhoeae* to human sperm. Infect. Immun. 9:897-902.
7. **Kellogg, D. S., I. R. Cohen, L. C. Norins, A. L. Schroeter, and G. Reising.** 1968. *Neisseria gonorrhoeae* II. Clonal variations and pathogenicity during 35 months in vitro. J. Bacteriol. 96:596-605.
8. **Koransky, J. R., R. W. Seales, and S. J. Kraus.** 1975. Bacterial hemagglutination by Neisseria gonorrhoeae. Infect Immun. 12:495-498.
9. **Novotny, P. and W. H. Turner.** 1975. Immunological heterogenicity of pili of *Neisseria gonorrhoeae*. J. Gen. Microbiol. 89:87-92.
10. **Ouchterlony, O.** 1958. Diffusion-in-gel methods for immunological analysis. Progr. Allergy 5:1-78.
11. **Punsalang, A. P. Jr., and W. D. Sawyer.** 1973. Role of pili in the virulence of *Neisseria gonorrhoeae*. Infect. Immun. 8:255-263.
12. **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.
13. **Waitkins, S.** 1974. Fimbrial hemagglutination by *Neisseria gonorrhoeae*. Br. J. Vener. Dis. 50:272-278.