Attachment Role of Gonococcal Pili

OPTIMUM CONDITIONS AND QUANTITATION OF ADHERENCE OF ISOLATED PILI TO HUMAN CELLS IN VITRO

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ABSTRACT Gonococcal pili facilitate attachment of virulent *Neisseria gonorrhoeae* to human cells. To characterize this attachment function, purified gonococcal pili isolated from four strains possessing antigenically distinct pili were radiolabeled with ^{125}I and used to measure the attachment of pili to various human cells in vitro. Human buccal and cervical-vaginal mucosal epithelial cells, fallopian tube mucosa, and sperm bound pili in greater numbers per μm^2 of surface area (1–10) than fetal tonsil fibroblasts, HeLa M cells, erythrocytes, or polymorphonuclear leukocytes. This cell specificity of attachment suggests a greater density of membrane pili binding sites on cells similar or identical to cells from natural sites of infection. The pili binding sites were quantitated as 1×10^4 per cervical-vaginal squamous cell.

Pili of all antigenic types attached equally to a given cell type, implying that the attachment moiety of each pilus was similar.

Attachment of gonococcal pili to human cells occurred quickly with saturation of presumed receptor sites within 20–60 min. Attachment was temperature dependent (37° > 20° > 4°C), and pH dependent (3.5 < 4.5 > 5.5 > 7.5). Attachment was inhibited by antibody to pili (homologous pili Ab > heterologous Ab). The extent of possible protection against gonococcal infection due to inhibition of pili-mediated attachment might prove limited as a result of the considerable antigenic heterogeneity among pili and the observation that blockage of pili attachment is maximal only with antibody to pili of the infecting strain.

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INTRODUCTION

The pili (fimbriae) of *Neisseria gonorrhoeae* presumably mediate the attachment of the gonococcus to human tissues. Previous evidence for an attachment role of pili has relied on the use of whole gonococci whose attachment to human cells was enhanced or occurred exclusively when that organism was piliated (1–8). Swanson (1) has reported that the presence of pili afford gonococci enhanced attachment to human amnion cells in vitro. The adherence of gonococci to human buccal mucosal cells (2), ecto- and endocervical epithelium, and fallopian tube mucosa (3), as well as exfoliated vaginal (4) and urothelial cells (5) is also enhanced by piliation. James-Holmquist et al. (6, 7) further implicated an attachment role of pili by demonstrating that antibody to purified gonococcal pili blocked the enhanced attachment of piliated gonococci to human sperm. The human fallopian tube has been used in perfusion culture by Ward et al. (8) to provide scanning electron microscope evidence of pili anchoring gonococci to the epithelial surface. The agglutination of human erythrocytes by the gonococcus has been correlated with the presence of pili by several investigators (2, 9–12). Recently, purified pili alone have been shown to be capable of direct erythrocyte agglutination (12). This agglutination can be blocked by antiserum to these purified pili, and the blockage reflects the antigenic heterogeneity of gonococcal pili (12).

For the gonococcus, the attachment of isolated pili to human cells other than erythrocytes has not been studied. A comparable model of an attachment moiety has been elucidated for *Escherichia coli* using an isolated mediator of adherence. Jones and Rutter (13) have characterized the adherence of cell-free K88 antigen to tissue from the small intestine of gnotobiotic piglets and the subsequent blockage of K88 positive *E. coli* adhesion with antibody to K88 antigen. In this

report we examined qualitatively and quantitatively the attachment of isolated ^{125}I-labeled gonococcal pili to various human cells and the effect of antibody to pili on that attachment.

METHODS

Strains of N. gonorrhoeae. Four strains of *N. gonorrhoeae* from patients with gonococcal urethritis were used. Three strains were from Dr. Douglas Kellogg (2686, F62) or Dr. Robert Arko (B) of the Center for Disease Control, Atlanta, Ga., and the fourth strain (33) was from Dr. Kenneth Johnston, The Rockefeller University, New York (Present address is Department of Microbiology, University of Texas, Dallas, Tex.). The pili isolated from these four gonococcal strains were antigenically distinguishable. This was shown by inhibition of direct hemagglutination, immunodiffusion precipitation, and radioimmunoassay (12), as well as rocket and two-dimensional immunoelectrophoresis (14–16).

All strains were grown on agar plates that contained GC medium base (Difco Laboratories, Detroit, Mich.) with 1% defined supplement (17) at 36.5°C in an atmosphere of 5% CO_2. Organisms were confirmed as gonococci by oxidase test, typical gram-negative diplococcal morphology, and sugar utilization reactions.

Purification of gonococcal pili. Solid media was inoculated with colony types 1 or 2 (18) of the strains used. After 18–20 h growth, the bacteria were harvested into ice-cold 10 mM Tris buffer (Trizma Base, Sigma Chemical Co., St. Louis, Mo.), pH 9.5, to an optical density of 1.5 at 560-nm wavelength. Bacterial suspensions were then sheared for 5 min by full-speed vortexing (Vortex Genie Mixer, Scientific Products Div., American Hospital Supply Corp., McGaw Park, Ill.). Organisms and debris were removed from the suspensions by centrifugation at 12,000 *g* for 10 min (Sorvall Superspeed RC2-B, Du Point Co., Instruments/Sorvall, Wilmington, Del.). The supernate was then centrifuged at 50,000 *g* for 50 min (Beckman Ultracentrifuge model L5-65, Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). The supernate was mixed well with an equal volume of 20% saturated ammonium sulfate and left at 4°C overnight. The ammonium sulfate suspension was centrifuged at 30,000 *g* for 10 min and the precipitate redissolved in distilled water. Protein determinations were performed on pili by the method of Lowry et al. (19) using bovine plasma albumin (Reheis Chemical Co., Chicago, Ill.) as a standard. The final yield was between 2–10 mg pili/10 g wet wt bacteria.

Criteria for purity of gonococcal pili. Pili were assessed for purity by electrophoresis in slab or disk gels consisting of 10% polyacrylamide, 0.3% bisacrylamide, and 0.1% sodium dodecyl sulfate (SDS).[1] Approximately 0.5–1.0 μg of pili labeled with ^{125}I was boiled for 5 min in 1% SDS, 1% 2-mercaptoethanol before applying to the disk gels. Upon completion of the electrophoresis, the gels were sliced in 2-mm segments and assessed for radioactivity. Approximately 10–50 μg of purified unlabeled pili were added to slab gels, and after electrophoresis the protein bands were fixed overnight in 5% trichloroacetic acid, stained for 3–5 h with 0.25% Coomassie Brilliant Blue (Sigma Chemical Co.) in 10% acetic acid containing 45% methanol, and destained in 7% acetic acid containing 10% methanol and Dowex 1-X8 anion exchange and Dowex 50W-X8 cation exchange resins (Bio-Rad Laboratories, Richmond, Calif.). Occasionally, radiolabeled preparations were electrophoresed over slab gels and the gels were dried and bands assessed by autoradiography. Pili preparations used in these studies gave a single protein peak in SDS polyacrylamide gel electrophoresis (SDS–PAGE; Figs. 2 and 3) (12). These preparations were not quantitated for carbohydrate, lipid, or nucleic acid content.

Transmission electron microscopy. Samples of purified gonococcal pili were examined as previously described (20, 21) using a JEOL, 100S transmission electron microscope operating at 100 kV.

Preparation of antisera to purified pili. Antiserum to purified gonococcal pili was prepared as described previously (12).

Purification of rabbit IgG immunoglobulin. The IgG from rabbit antiserum to purified pili was concentrated by repeated precipitation at a 1.75 M concentration of ammonium sulfate followed by DEAE chromatography at pH 6.6 (21). The purity of the IgG preparations was assessed by immunoelectrophoresis (22) using goat antiserum to rabbit IgG or whole serum (Miles Laboratories Inc., Miles Research Products, Elkhart, Ind.). Pure IgG preparations gave single precipitin lines with anti-whole rabbit serum, and the position of the precipitate corresponded to that produced by antiserum to rabbit IgG.

Quantitation of IgG specific for pili. To determine the relative quantity of IgG specific for pili, purified IgG preparations were run against homologous pili in rocket immunoelectrophoresis (15, 16). Glass microscope slides (7.5 × 2.5 cm), which were precoated with 1.0 ml 1% agarose and dried at 37°C, were coated with 2.5 ml of 0.5% agarose containing IgG diluted in veronal buffer (0.083 M barbital sodium, 0.0166 M barbital [Fisher Scientific Co., Pittsburgh, Pa.], pH 8.6). Antigen wells were made in the solidified agarose using a punch measuring 2-mm inside and 4-mm outside diameter. Pili solutions were standardized to 612 μg/ml by the addition of phosphate-buffered saline, pH 7.3, containing 1% by weight bovine plasma albumin (1% PBSA), and then sonicated for 5 min to insure pili fragments capable of migrating through the agarose gel. Further dilutions were made in 1% PBSA. The quantity of pili added to antigen wells ranged from 122 ng to 3.06 μg. Constant volumes and weights of pili were used to compare IgG specific for the homologous pili type in each of the IgG preparations. Electrophoresis was conducted in veronal buffer at 6 V/cm to completion (120 min) or at 2 V/cm overnight with antigens migrating toward the anode. After electrophoresis, slides were rinsed overnight in 5 mM TES (Calbiochem, San Diego, Calif.) containing 0.15 M NaCl, pH 7.3 at 4°C. Slides were rinsed in distilled water for 10 h and then dried completely at 37°C. slides were stained with 0.5% Coomassie Brilliant Blue in 45% ethanol and 10% glacial acetic acid. Destaining was done with the above solvent. The relative quantity of the pili-specific IgG in different preparations was calculated as the ratio of dilutions of the two IgG preparations that each gave rocket precipitates equal in area when run against equal amounts of their respective, homologous pili (16). The reciprocal of a dilution was termed the titer, and these titers were used to compare relative quantities of antibody. (For example, if two IgG preparations, varying in titer by 20-fold, run against equal amounts of homologous pili gave rocket precipitates equal in area, the IgG preparation with the higher titer was considered to contain 20 times more pili-specific IgG.)

Radiolabeling of gonococcal pili with ^{125}I. Pili were labeled either by the chloramine-T procedure of Greenwood et al. (21, 23) or by conjugation to a ^{125}I containing

[1] *Abbreviations used in this paper*: BSA, bovine serum albumin; DNP, 2,4-dinitrophenol; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline (10 mM dibasic sodium and 3 mM monobasic potassium phosphate buffer, pH 7.3, 0.12 M NaCl); 1% PBSA, PBS containing 1% by weight bovine plasma albumin; PMN, polymorphonuclear leukocytes; RBC, human erythrocytes; SDS, sodium dodecyl sulfate.

acylating agent (iodinated 3-[4-hydroxyphenyl] propionic acid *N*-hydroxysuccinimide ester; Pierce Chemical Co., Rockford, Ill.) using the procedure of Bolton and Hunter (24). Pili preparations were sonicated for 30 s in a bath sonicator (Heat Systems-Ultrasonics, Inc., Plainview, N. Y.) before labeling. Specific activities obtained with chloramine-T and Bolton and Hunter procedures were approximately 1,000 cpm/ng and 250 cpm/ng, respectively.

Assessment of antigenicity of radiolabeled pili. ^{125}I-Labeled pili used in these studies were shown to be antigenic by radioimmunoassay (12, 21, 25) and agar gel immunodiffusion (12).

Human cells tested—collection and preparation

All cells were washed three times using ≥ 20 vol phosphate-buffered saline, pH 7.3 (PBS) per volume pellet by centrifugation (model PR-2, International Equipment Co., Div. Damon Corp., Needham Hts., Mass.) and were tested in a 25% (vol cells/total vol) suspension, unless otherwise stated, in PBS. Enumeration of human cells/mm^3 was made by direct microscope count in a Spencer Improved Newbauer Hemacytometer (American Optical Corp., Scientific Instrument Div., Buffalo, N. Y.). Cell viability was monitored by exclusion of 0.016% trypan blue (Grand Island Biological Co., Grand Island, N. Y.). All nontissue culture cells (except erythrocytes) were tested within 6 h of obtaining the cell specimen.

Erythrocytes (RBC). Blood group O and A Rh+ human blood was obtained in heparinized tubes, and the cells were separated from the serum by low-speed centrifugation and washed three times with PBS. RBC were then used immediately, or a portion was stored at 4°C for up to 2 wk in an equal volume of Alsever solution (26). RBC stored in Alsever solution, after preparing as above, behaved comparably to freshly obtained RBC (12).

HeLa M cells. Suspension cultures of HeLa M cells were grown at 37°C in spinner flasks to a concentration of approximately 8×10^5 cells/ml. The cells were separated from the growth media (Auto-pow minimal essential medium for suspension culture, Flow Laboratories, Inc., Rockville, Md.; 10% fetal bovine serum, Microbiological Associates, Walkersville, Md.; 2 mM L-glutamine, Calbiochem, with 100 U/ml penicillin and 100 mg/ml streptomycin added, pH 7.2) by low-speed centrifugation, washed and resuspended. Alternatively, monolayer cultures of HeLa M cells grown at 37°C with the substitution of Eagle's modified minimal essential medium, (Grand Island Biological Co), in 32-oz glass flasks were used. Cells were removed with 1–2 ml 0.05% trypsin (Difco Laboratories). After removal, 10 ml of GKN solution (0.8% NaCl, 0.04% KCl, and 0.1% glucose) containing 10% fetal bovine serum was added to inhibit the trypsin, and the cells were processed as for suspension cultures after 1 h at 37°C.

Fetal tonsil fibroblasts. Monolayer cultures of fetal tonsil diploid cells (27) (19–25th passage) grown in 32-oz glass flasks with the same media as HeLa monolayers at 37°C were harvested by the addition of 1–2 ml 0.25% trypsin. The trypsinized cell suspensions were diluted with 10–15 ml GKN solution (0.8% NaCl, 0.04% KCl, and 0.1% glucose) containing 10% fetal bovine serum and incubated at 37°C for 60 min before washing and resuspension for use. Where 2,4-dinitrophenol (DNP) was used as a metabolic poison, final fetal tonsil cell dilutions were made in 1 mM DNP (28) in PBS containing 1.0% ethanol. These cells, as well as controls diluted only in PBS, were incubated for 30 min at 37°C and then used immediately in attachment experiments without removing the DNP.

Cervical-vaginal epithelial cells. Mixtures of cervical and vaginal epithelial cells were obtained from uninfected women examined at a Seattle Venereal Disease or Women's clinic. 10 ml of nonbacteriostatic normal saline was inserted into the posterior vaginal vault, and a cotton swab was used to gently remove cells from the region of the cervical os. This was collected and the cells were separated by low-speed centrifugation. Washing removed most adhered bacteria.

Sperm. Samples of human semen were furnished by males who denied any previous or present gonococcal infection. Seminal fluids were diluted to 5 ml with PBS and centrifuged at 500 *g* for 10 min. The supernate was discarded and the pelleted sperm were washed three times and resuspended to a concentration of 12.5% (vol/vol) for use as described above.

Buccal mucosal cells. Epithelial cells were gently scraped with wooden tongue depressors from the buccal mucosa of healthy individuals. Buccal cells from several individuals were suspended in PBS, pooled, and concentrated by low-speed centrifugation. Cells were washed three times and resuspended as described above for use.

Human fallopian tube mucosa. Sections of human fallopian tube mucosa between the ampulla and infundibulum, excised from women undergoing tubal ligations by laparoscopy, were obtained from specimens destined for pathological examination. Fallopian tube sections were kept moist in sterile medium 199 (Grand Island Biological), cut longitudinally to expose the mucosal surface, and then cut into 0.5-cm^2 strips. ^{125}I-Pili were added directly to the exposed surface, and separate strips, after 0, 15, or 30 min at 37°C, were washed five times in PBS, vortexed in PBS, and then counted for radioactivity. The surface area exposed to pili was estimated to be between $1-2 \times 10^8$ μm^2 total/strip.

Polymorphonuclear leukocytes (PMN). Blood group O Rh+ human blood was obtained in heparinized syringes and added to an equal volume of 3.0% Dextran T-500 (Pharmacia Fine Chemicals, Piscataway, N. J.) in 0.9% NaCl. The RBC were allowed to settle at room temperature, and the supernate was withdrawn and centrifuged at 250 *g* for 5 min at 4°C in a clinical centrifuge (International Equipment Co.). The pellet of PMN cells was mixed vigorously on a Vortex mixer with 10 ml of ice-cold 0.2% NaCl for 25 s to hypotonically lyse RBC. Immediately, 10 ml of ice-cold 1.6% NaCl was added, and the mixture recentrifuged as above. This last step was repeated if any RBC remained in the pellet. The cells were then resuspended in PBS, washed once, counted, and adjusted to a concentration of 12.5% (vol/vol). Where sodium fluoride (NaF) was used as a metabolic poison and an inhibitor of phagocytosis, final PMN dilutions were made in 0.02 M NaF (28) in PBS. These cells, as well as controls diluted only in PBS, were incubated for 30 min at 37°C and then used immediately in attachment experiments without removing the NaF.

Calculation of cell surface area. Values for the surface area of the cells tested were, where possible, obtained or calculated from the literature cited in Table I. Otherwise, surface areas were estimated from values of the "average profiles" and circumferences of the cells obtained by morphometry (29). Morphometry was performed by using cell suspensions that were first fixed for 1 h in 1.5% glutaraldehyde in 0.11 M 2,4,6-collidine (Sigma Chemical Co.) buffer, pH 7.4, and then washed in 2,4,6-collidine buffer. Buccal, cervical-vaginal, and fetal tonsil cell preparation wet-mounts were examined using phase contrast microscopy (Zeiss Ultraphot II, Ober Kochen, Wuerttenberg, W. Germany, ×400) and a Zeiss integrating eyepiece, (type I, six

horizontal lines, 25 points) standardized with a stage micrometer. Rough estimates of cell diameter were first obtained by directly measuring 10–20 random cells of each. The average profile (*AP*, mean linear intercept) (29) was then calculated for 172 stained (methylene blue; Matheson, Coleman & Bell, East Rutherford, N. J.) buccal cells and 152 and 178 stained cervical-vaginal cells using the number of cell intersections with the integrating eyepiece horizontal lines and the formula (29): AP = total length of lines in micrometer state × total number of fields observed/total number of intersections × 2. In addition, planimetry (29) was performed on phase contrast micrographs (×200) of unstained wetmounts of buccal (54 cells measured), cervical-vaginal (74 cells), and fetal tonsil cells (20 cells) using a map measure (Keuffel and Esser Co., Morristown, N. J.) to obtain the mean cell circumference. The average diameter of a buccal cell was calculated by these three methods to be 53.0 μm (average of 50 and 56 μm) and that of a cervical-vaginal cell to be 47.0 μm. This was in agreement with prior rough estimates and with previous investigations (40–50 μm) (30). The calculated average fetal tonsil cell diameter was 21.1 μm.

To calculate cell surface areas, it was assumed that both buccal and cervical-vaginal epithelial cells are cuboids flattened in shape (30) with an average width of 4 μm. Thus, the formula for the surface area of a cylinder (2 [πr^2] + width × circumference) was used to calculate surface area for these two cells (Table I). The surface area of fetal tonsil cells was calculated by πd^2 assuming that these cells are spherical in suspension. It is possible that the complex nature of the surface of, at least, the buccal cell, which contains many microridges, may result in some underestimation of the surface area and overestimation of the number of pili binding per μm^2 (Table I).

Assay of attachment of ^{125}I-labeled pili to human cells. ^{125}I-Labeled pili were diluted in 1% PBSA and centrifuged at 10,000 *g* for 10 min before use in the assay to remove material that would pellet during the centrifugation step of the subsequent assay. Labeled pili diluted in 1% PBSA (50 μl) was added to an equal volume of 1% PBSA. To this, 0.2 ml of human cell suspension (25% vol/vol, unless otherwise specified; see Table I) was added, and solutions were mixed well. The amount of pili added to reaction mixtures ranged from 110 to 1,300 ng. The pili-cell suspensions were incubated with shaking at 37°C in a water bath or alternately at

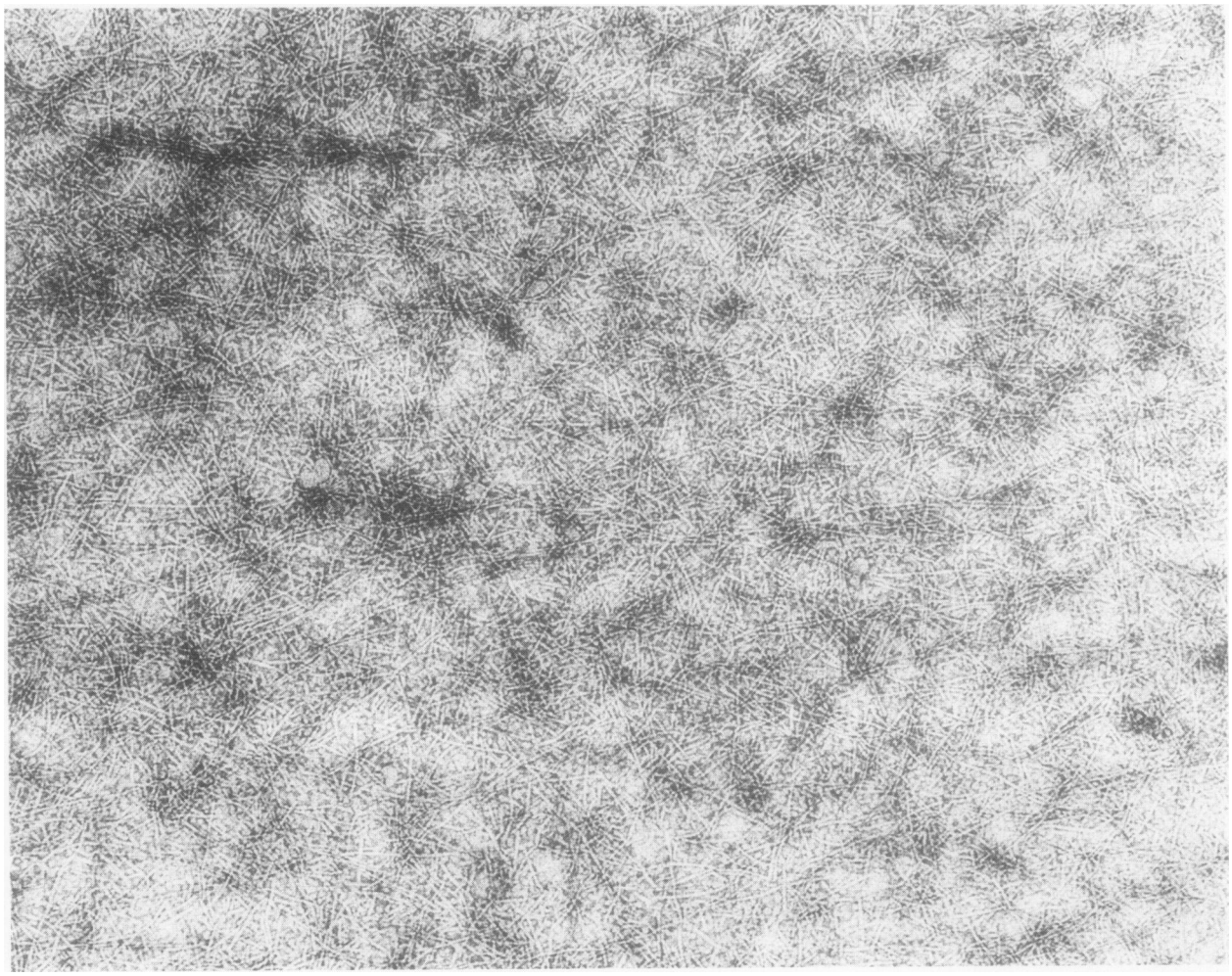

FIGURE 1 Electron micrograph of purified gonococcal pili from strain F62. The pili preparation (27:1 purity [pili peak: background radioactivity], SDS-PAGE) was stained with 1% potassium phosphotungstic acid, pH 7.0. ×63,500.

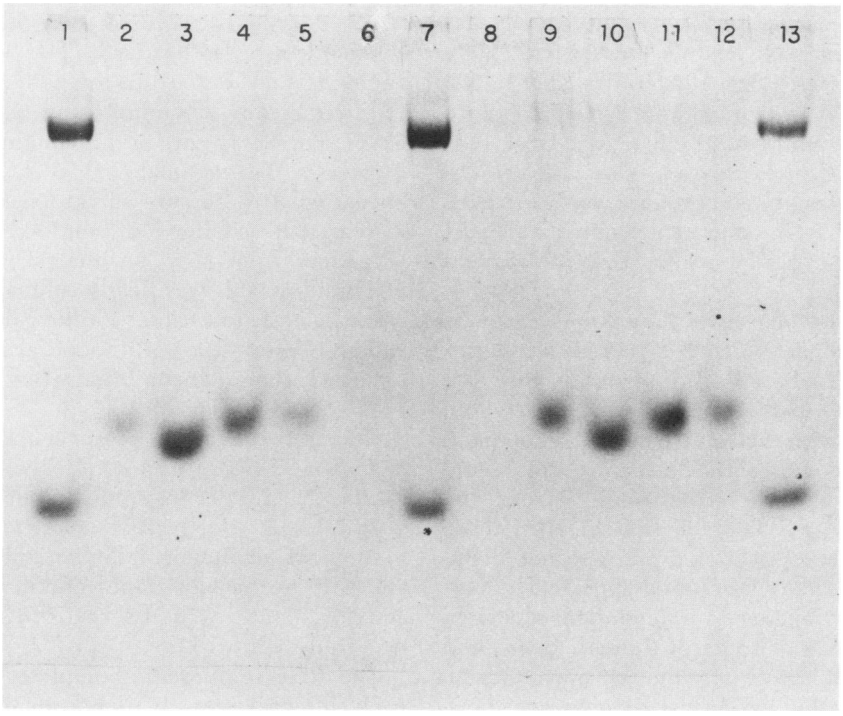

FIGURE 2 SDS polyacrylamide slab gel electrophoresis of gonococcal pili from strains B, F62, 33, and 2686, and molecular weight markers. Protein staining is with Coomassie Brilliant Blue. Wells 1, 7, and 13 = BSA (mol wt = 66,800) and cytochrome *c* (12,700); wells 2 and 9 = B pili (18,400); wells 3 and 10 = F62 pili (16,500); wells 4 and 11 = 33 pili (18,250); wells 5 and 12 = 2686 pili (19,000); wells 6 and 8 = trypsin (24,000).

room temperature or at 4°C. 10-μl samples were removed from the pili-cell mixture at varying time intervals after initial mixing together of cells and pili, and added to 0.25 ml of PBS in a 0.4-ml plastic microfuge tube (Beckman Instruments, Inc.). The microfuge tubes were then centrifuged for 1 min in a Beckman microfuge (model 152), 95% of the supernate was withdrawn, the pellets were washed with 0.2 ml of PBS and recentrifuged for 5 min. The supernate was withdrawn and the portion of the microfuge tube containing the pellet was cut off and measured for radioactivity in a gamma spectrophotometer (Packard Instrument Co., Inc., Downers Grove, Ill.). This procedure was conducted for each 10-μl sample of the pili-cell mixture. Duplicate samples were assayed for each time interval. Background radioactivity was subtracted from all data. As a control, 10 μl of labeled pili alone was processed as described for pili-cell mixture samples and gave final counts equivalent to background. The percent of added pili that bound cells was 7–18%

Inhibition of ^{125}I-pili attachment by antibody to pili. Where IgG purified from rabbit antiserum to purified pili was used to inhibit attachment, 50 μl diluted, radiolabeled pili was incubated with an equal volume of IgG diluted in 1% PBSA, or 1% PBSA alone in controls, for 15 min at room temperature. The attachment experiments were then conducted as described at 37°C. Percent inhibition at 20 and 40 min was calculated as:

$$\frac{\text{observed attachment (cpm) without antibody} - \text{observed attachment (cpm) with antibody}}{\text{observed attachment (cpm) without antibody}} \times 100.$$

Characterization of radiolabel adhering to human cells. Buccal mucosal cells incubated with labeled pili at 37°C for 1 h were pelleted in a microfuge tube, washed thoroughly, and then assessed for pili by electrophoresis in SDS-PAGE as described above (Fig. 3 B).

Quantitation of pili/cell ratios

The specific activity (counts per minute/nanogram) of each preparation of labeled pili was determined. Using this, Avagadro's number, and the molecular weight for the size of a pilus that attached calculated to be 2,000,000 (21; Fig. 1), a direct ratio of pili to radioactivity could be determined. The concentration of human cells in each pili-cell mixture was obtained using a hemacytometer. The ratio of the number of pili adhering to each cell was then determined according to the formula: pili/cell (cpm [pili] attached to cells × number of pili/cpm)/(concentration of cells tested in sample [per mm^3]) × (1/vol of sample [mm^3]).

RESULTS

Assessment of purity of gonococcal pili. Though not a criteria of purity, electron micrographs of negatively stained, purified gonococcal pili from strain F62 showed abundant pili and occasional vesicular particles present with little morphologically recognizable contaminants (Fig. 1). Fig. 2 shows the slab SDS 10% polyacrylamide gel patterns of purified pili from strains F62, B, 33, and 2686. A single major protein

peak was found in each case. The subunit molecular weights of pili from each strain were approximately 18,400 (B pili), 16,500 (F62), 18,250 (33), and 19,000 (2686). Radiolabeled pili preparations used in these experiments were examined by radioactive SDS-PAGE disk gels. The purity, expressed as a ratio of the pili protein to any contaminant protein, was 7:1 (F62—one experiment), 8:1 (B—one experiment), and 10:1 or more (all other experiments for F62, B, 33, and 2686 pili).

Attachment of ^{125}I-labeled pili to human cells. Fig. 3 compares the radioactive SDS-PAGE disk gel patterns for an original labeled pili preparation (Fig. 3A, strain B) and for the washed pellet of buccal mucosal cells after reacting with the same labeled pili preparation (Fig. 3B). The radioactive peak present in the washed cell pellet is of the same subunit size as the dominant radioactivity peak in the original purified pili peak, indicating that radiolabeled pili, and not a contaminant, has attached to the epithelial cells. The possibility that pili may aggregate independent of attachment in the presence of some of the cell types, and thus sediment with the cells although not attached, cannot be completely excluded. However, transmission electron microscope examination of 1% phosphotungstic acid stained (pH 7.0) pili-cell pellets showed no evidence of extracellular pili clumping.

Effect of cell type and pili source on attachment. Pili isolated from four different gonococcal strains attached equally well, as did pili labeled by the activated ester procedure (24), which labels free amino groups (Fig. 4). In this latter case, the binding of fewer counts per minute can be explained by the lower specific activity of the labeling. This figure also illustrates that radiolabeled BSA did not attach to buccal cells. To assure saturation, the data in Fig. 4 were treated as following pseudo-first order kinetics. It was clear from such an analysis that saturation of pili binding sites was nearly achieved in most experiments. Consistent values for the calculated number of cell sites present, or pili bound at infinite time, were obtained for similar conditions (B = 395, F62 = 400, 33 = 425, and 2686 = 500).

Human buccal and cervical-vaginal mucosal epithelial cells bound ^{125}I-pili most quickly. Fetal tonsil fibroblasts attached pili less quickly but faster than human sperm, HeLa M cells, RBC, and PMN. Pili attachment to sections of human fallopian tube also proceeded similarly to the most rapid group. Of the total radioactivity bound by the tissue, 70% of the binding had occurred within 15 min.

The time required to complete pili attachment to each cell was inversely proportional to: number of pili/total cell surface area (Table I). For high pili/cell surface area ratios, ≥ 80% of attachment of pili occurred to buccal and cervical-vaginal cells within 20 min.

To test whether this binding was reversible, a ^{125}I-pili-buccal cell mixture was incubated for 15 min at

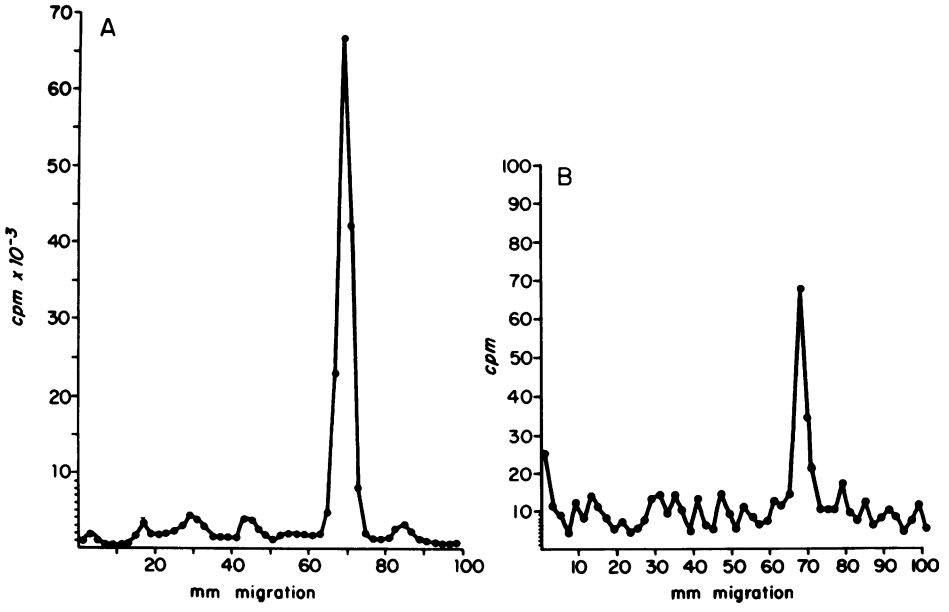

FIGURE 3 Radioactive SDS polyacrylamide disk gel electrophoresis of gonococcal pili or of a mixture of pili and buccal cells. (A) Disk gel pattern for an original, ^{125}I-labeled pili preparation from strain B, demonstrating a single major peak correlating with the subunit molecular weight of pili. (B) Disk gel pattern for the washed pellet of buccal mucosal cells after reacting with ^{125}I-labeled B pili.

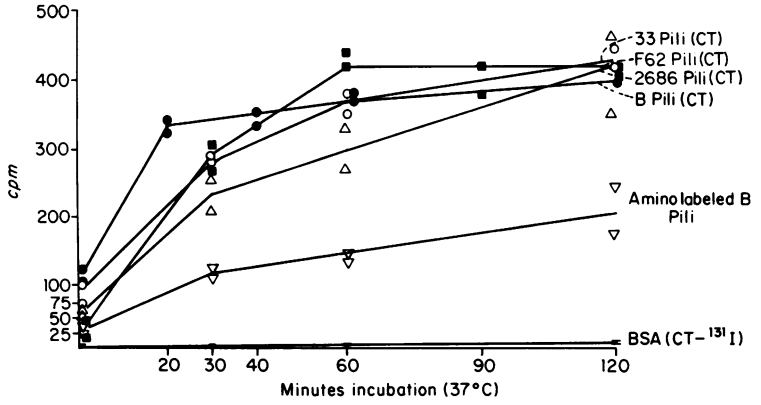

FIGURE 4 Comparison of the attachment of ^{125}I-labeled pili from four different gonococcal strains labeled by the chloramine-T procedure (CT), ^{125}I-pili labeled by the Bolton and Hunter procedure (Amino labeled), and ^{131}I-BSA to buccal mucosal cells at 37°C. (○), 33, (■), F62, (△), 2686, (●), B, (▽), Amino labeled B, (——), BSA. CPM, ^{125}I-pili or ^{131}I-BSA counts per minute contained in the cell pellet. The specific activities (counts per minute/nanogram) of the labeled pili and the amount (nanogram) added to the reaction mixture, respectively, were: 33—1,200,113; F62—520,254; 2686—770,170; B—1,040,125; and amino labeled B—237,373. Approximately 8×10^5 cells were added to each reaction mixture.

37°C after which time the cells were washed in PBS and a 100-fold excess of unlabeled homologous pili was added. Upon continued incubation, no appreciable decrease occurred in cell-bound ^{125}I-pili within 30–60 min, indicating a very low dissociation constant, and a stable bond between pili and the cell surface pili binding site.

Trypan blue-stained smears of buccal mucosal and cervical-vaginal epithelium used in these experiments revealed that most (>90%) cells were nonviable, presumably near desquamation at the time of collection. The ability of pili to bind fetal tonsil fibroblasts, which were greater than 70% viable before use in attachment experiments, provided the opportunity to investigate an energy requirement, if any, for binding. Interestingly, the addition of DNP, an uncoupler of mitochondrial oxidative phosphorylation (ATP synthesis) did not inhibit subsequent pili attachment in concentrations of 1 mM when added to fetal tonsil cells and incubated at 37°C for 30 min. This suggested that attachment does not require cellular energy. Similarly, when PMN were incubated with an inhibitor of glycolysis, NaF (20 mM, 30 min at 37°C), no demonstrable inhibition of pili attachment occurred.

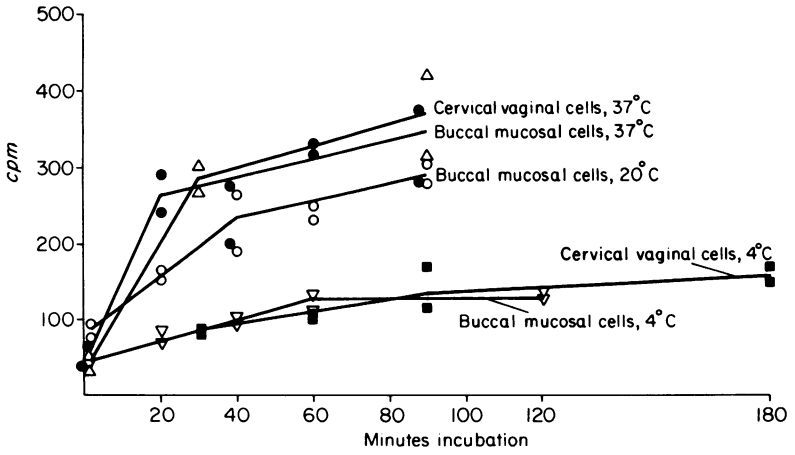

FIGURE 5 Temperature dependence of binding of ^{125}I-labeled gonococcal pili from strain B to buccal and cervical-vaginal epithelial cells. Pili-cell mixtures were incubated at temperatures of either 4°, 20°, or 37°C. (△), cervical-vaginal cells, 37°C; (●), buccal cells, 37°C; (○), buccal cells, 20°C; (■), cervical-vaginal cells, 4°C; (▽), buccal cells, 4°C. The specific activity of the labeled pili was 237 cpm/ng. Each reaction mixture contained 373 ng labeled pili and 1.05×10^6 cervical-vaginal or 7.6×10^5 buccal cells.

Effect of temperature, pH, and ions on attachment. The attachment of pili to human cells occurred at temperatures of 4°–37°C. Optimal attachment, as demonstrated by human buccal mucosal and cervical-vaginal cells in Fig. 5, occurred at 37°C followed by 20° and 4°C in decreasing order. The optimum pH for pili attachment is 4.5. At this pH, attachment is three- to fourfold greater than the amount at pH 7.4 (Fig. 6). This pH optimum is relatively sharp in either direction. Also, 0.1 mM ferric ion, pH 7.2, enhanced pili attachment approximately twofold on repeated testing. Enhanced attachment in the presence of cations occurred with 0.1 mM $Fe^{+++} > 0.1$ mM $Fe^{++} >$ 1mM Ca^{++}, or 1 mM Mg^{++}. Heating ^{125}I-pili at 85°C for 1 h or exposure to UV irradiation for 2 h (31) reduced subsequent attachment (Fig. 6).

Pili/cell ratios for human cells tested. Table I summarizes the number of pili binding per cell for each cell type tested. Lowest cell concentrations were used to maximize pili binding per cell. An average molecular weight of pili attaching to cells was estimated to be 2×10^6 (21; Fig. 1). Thus, the maximum number of pili adhering to buccal mucosal cells was between 25,000–50,000 pili/cell (Table I). For cervical-vaginal cells the number was approximately 6,000–13,000 pili/cell. Fetal tonsil cells attached between 500 and 1,400 pili whereas human sperm, RBC, PMN, and HeLa cells were able to bind between 10 and 370 pili/cell. HeLa attachment, which is the highest of this last group of cells (geometric mean = 189), was similar for cells harvested from suspension and monolayer cultures. For the latter, the number of pili/cell was between 140–190, while the range for suspension cultures was 70–370. Not shown are data from human fallopian tube mucosa, which was used in square strips to which pili were added. The increase in tissue bound radioactivity indicated that pili attach avidly and rapidly to fallopian tube mucosa. Pseudo-first order kinetics analysis of pili attachment to the human cells in Table I demonstrated that saturation was nearly achieved except for sperm, PMN, and HeLa cells. These were between 50–75% saturated. Therefore, the pili binding sites per cell given for these three cell types may be low but accurate within a factor of two.

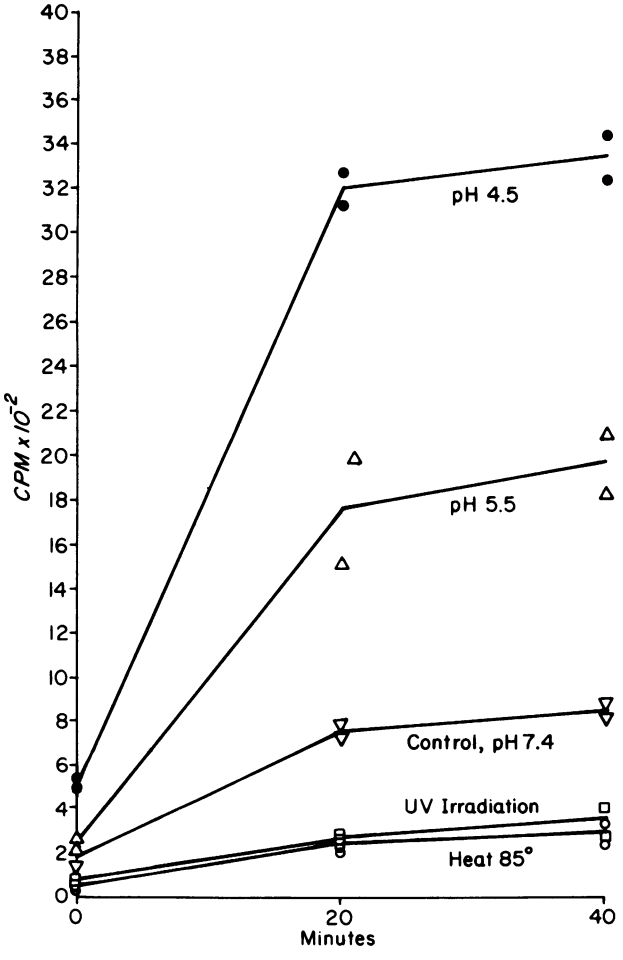

FIGURE 6 Attachment of ^{125}I-gonococcal pili from strain F62 to human buccal mucosal cells. Pili either untreated or treated with UV irradiation for 2 h (□), or heat at 85°C for 1 h (○) were reacted with cells at 37°C at pH 4.5 (●), 5.5 (△), or 7.4 (▽). Each reaction mixture contained approximately 1,300 ng labeled pili (375 cpm/ng) and 7.8×10^5 cells.

Correction for cell surface area (Table I) reveals that in terms of pili per μm^2, pili binding sites are most common per unit area on buccal and cervical-vaginal epithelium and sperm. These cells bound 1–10 pili/μm^2. Values for fetal tonsil fibroblasts indicate binding sites are less common (0.6–1.0 pili/μm^2). The number of pili per surface area for RBC, PMN, and HeLa cells lies within the lowest range (0.1–0.6 pili/μm^2). Thus, gonococcal pili binding sites per unit area are approximately 10- to 20-fold more common on buccal and cervical-vaginal cells and sperm as compared to the other cells tested. The total surface area of fallopian tube mucosa exposed to added pili was roughly estimated to be between $1–2 \times 10^8$ μm^2. To this, 6.11 $\times 10^8$ pili were calculated to attach. This is equivalent to 3.06–6.11 pili/μm^2, which is in the highest range.

Fig. 7 illustrates attachment of ^{125}I-labeled 2686 or B pili to varying concentrations of buccal mucosal cells at 37°C, in an attempt to saturate cell surface pili binding sites. Pili per cell ratios were increased, and more rapid saturation of binding sites was observed with more dilute concentrations of cells (Fig. 7). Analysis of this data by pseudo-first order kinetics also showed that saturation had been achieved. Reducing the cell concentration allowed verification of the calculated total number of cell binding sites present. For example, in B pili attachment (Fig. 7) to buccal cells used in concentrations of 6.2, 12.5, 18.7, and

TABLE I
Pili/Cell Ratios 37°C

Human cell tested	Concentration range ($\times 10^{-3}$) per mm³ / total number ($\times 10^{-5}$) for cells tested	Estimated surface area/cell	Number of pili attaching/cell** geometric mean / range	Pili/μm^2	
				Geometric mean	Maximum
		(μm^2)			
Buccal mucosal epithelium	0.65–2.7 / 1.9–8.0	5,116*	25,100 / 8,680–54,100	4.91	10.6
Cervical-vaginal epithelium	1.7–6.8 / 2.6–17	4,056*	10,100 / 6,230–13,300	2.48	3.27
RBC	160–1,850 / 470–5,540	138‡	15.6 / 6.42–67.0	0.113	0.486
PMN	17–140 / 49–410	237§	68.3 / 54.7–88.4	0.288	0.373
Sperm	200–250 / 600–760	84‖	86.4 / 15.2–305	1.03	3.63
HeLa "M" cells	8.7–87 / 26–260	600¶	189 / 74.3–367	0.315	0.612
Fetal tonsil fibroblasts	6.5–26 / 19–78	1,400*	824 / 515–1440	0.589	1.03

* See Methods (calculation of cell surface area).
‡ Reference 32.
§ Calculated from a volume of 343±9 μm^3 (33) for PMN from normal populations in suspension.
‖ Surface area of head ≈ 50 μm^2; midpiece ≈ 6 μm^2; tail ≈ 28 μm^2 (personal communication from Dr. Ann N. James).
¶ Reference 29; personal communication from Dr. E. S. Boatman.
** Summary of 4–10 experiments. Higher values were obtained when lowest cell concentrations were used to maximize pili binding per cell. Pili binding to sperm, PMN, and HeLa cells produced 50–75% saturation of binding sites. Complete saturation of pili binding sites was reached for some experiments with all other cell types.

25%, the number of sites present was calculated to be 650, 930, 1,100 and 1,900, respectively. Dividing these values by the percent cell concentration used gave approximately constant values (104, 78, 61, and 76, respectively) with a mean of 78±19. This constancy is expected at saturation.

Similar experiments to that shown in Fig. 7 were also performed using human RBC. Pili per cell ratios increased from 30.5 with 25% cells to 67 pili per cell with 2.5% RBC. Also, saturation of binding sites was more quickly achieved with more dilute cells.

Effect of antibody to pili on the attachment of ^{125}I-pili. The inhibition of pili attachment by IgG specific for pili is summarized in Table II. Pili from strains 2686 or B were tested for inhibition of attachment to buccal cells by IgG to 2686, B, or F62 pili. Quantitative rocket immunoelectrophoresis (see Methods) indicated the following relative values for IgG specific to homologous pili in each IgG preparation: 2686/B = 18/1; 2686/F62 = 8.2/1; F62/B = 2.2/1. Thus, if IgG antibody to F62 pili was equal to B pili antibody at blocking B pili attachment, 2.2 times as much of the B pili IgG preparation as compared with the F62 pili IgG would be required to produce equal inhibition of attachment. However, if only 1.5–2.0 times more of the IgG antibody preparation to B pili than IgG to F62 pili were required to produce equivalent inhibition, it would indicate stronger inhibition of pili attachment by IgG to the homologous pili type. To express how many fold more antibody is required of the heterologous preparation to produce the same level of inhibition of attachment as the homologous antibody to pili, the following ratio was used: amount of heterologous antibody observed to produce 40% inhibition of attachment/amount of heterologous antibody predicted to produce 40% inhibition of attachment. Because the amount of antibody is inversely proportional to the titer of antibody, this formula is equal to:

$$\frac{Tp}{To} = \frac{\text{titer predicted of heterologous IgG to produce 40\% inhibition}}{\text{titer observed of heterologous IgG to produce 40\% inhibition}}.$$

In each instance, IgG antibody to the homologous pili

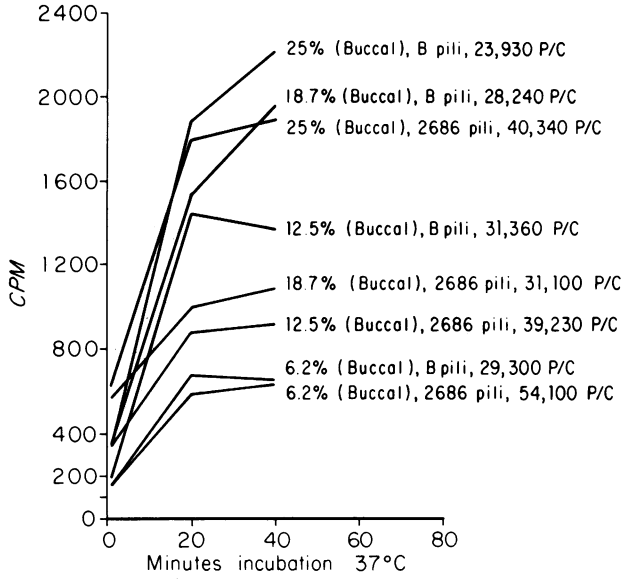

FIGURE 7 Relationship between cell concentration and attachment of ^{125}I-labeled gonococcal pili from strains 2686 (547 cpm/ng) and B (1,075 cpm/ng). Varying concentrations of buccal cells (6.2-25% vol/vol) were used in attachment assays in which ^{125}I-pili and 1% PBSA were added. The 25% concentration of buccal cells represents 7.75×10^5 cells added to the reaction mixture. Each reaction mixture contained 575 ng of labeled 2686 pili or 653 ng of labeled B pili. P/C, the calculated number of pili bound per cell in each experiment.

type was more effective than IgG to heterologous pili, and the observed titer of heterologous antibody producing 40% inhibition of attachment was lower than the predicted titer (Table II), and normal rabbit IgG produced insignificant inhibition (8%).

DISCUSSION

By utilizing radiolabeled gonococcal pili, we have confirmed the attachment role of pili and elucidated specific features of this attachment to human cells. The attachment of pili occurs rather rapidly and was similar for the antigenically distinct pili isolated from the four strains used. Our results suggest pili possess specificity for certain cell surfaces. These data suggest that more binding sites capable of attaching pili exist on the surface of cervical-vaginal epithelial cells, sperm, and probably fallopian tube mucosa than fetal tonsil fibroblasts, RBC, PMN, and HeLa M cells. This is consistent with the results of Tebbutt et al. (3) who found that piliated gonococci adhered better than nonpiliated organisms to human endocervix, ectocervix, and fallopian tube mucosa, but not to human bronchial mucosa or epithelial surfaces of guinea pig tissues. The number of pili attaching to a single buccal or cervical-vaginal cell (i.e. pili binding sites) is between $1-5 \times 10^4$ assuming that the size of an attaching pilus is 2×10^6 daltons (Table I). In contrast, only 6.4–67 pili attached per human RBC in this study. This level of attachment of pili to cervical-vaginal cells is comparable to the number of receptor sites for adenovirus intact virions per KB or HeLa cell (10^4) and of adenovirus tail fibers alone (10^5) (34, 35). For insulin binding human fat cells, Cuatrecasas (36) has reported 10^4 molecules per cell, whereas 6×10^3 human chorionic gonadotropin molecules will saturate a single Leydig cell (37) and 4×10^3 sites for human growth hormone are found on cultured lymphocytes (38). Correction for the surface area of these cells provides a measure of the frequency of pili binding sites (pili/μm^2; Table I). Thus, the number of pili attaching per μm^2 to human cervical-vaginal or buccal mucosal cells is 2.5–10.0 (Table I). The insulin receptor density per fat cell is estimated by Cuatrecasas (39) to be about 10 sites/μm^2. Jarett and Smith (40) have calculated a higher density ranging from 22–100 sites/μm^2 by two separate techniques. It is perhaps significant that our results find the most attachment of pili and, corrected for surface area, the most binding sites in cells that are histologically the most similar to the actual sites of human gonococcal infection (cervical-vaginal and buccal cells, sperm, and fallopian tube

TABLE II
Inhibition of ^{125}I-Pili Attachment to Buccal Cells by Rabbit IgG Antibody Prepared against Purified Pili

IgG to pili	Pili for attachment	IgG titer producing 40% inhibition	Tp*§ / To‡
B	B	30.0	1
F62	B	11.5 Tp = 66.0	5.7
2686	B	81.0 Tp = 540	6.7
2686	2686	450	1
B	2686	19.0 Tp = 25.0	1.3
F62	2686	8.0 Tp = 54.9	6.9

^{125}I-Pili were incubated with IgG diluted in 1% PBSA, or 1% PBSA alone in controls, at room temperature for 15 min before addition of buccal cells. Percent inhibition (see Methods) was calculated at 20 and 40 min of incubation at 37°C.

* Tp, Titer of the original IgG preparation predicted to produce 40% inhibition based upon the amount of antibody specific to homologous pili present (see Results).

‡ To, Titer observed to produce 40% inhibition.

§ Tp/To, strength of homologous pili IgG at producing 40% inhibition relative to the identical quantity of heterologous pili IgG.

mucosa). A similar cell tropism exists in the preferential binding of adenovirus fibers to KB and HeLa cells in culture as opposed to L cells or isolated plasma membranes from human lymphocytes (35). KB and HeLa cells will support a lytic infection by adenovirus but L cells will not. Similarly, insulin binds maximally to fat and liver cell membranes (36, 41, 42) whereas luteinizing hormone demonstrates highest affinity to Leydig cells of the rat testis (43) and in the rat ovary to corpora lutea and interstitial tissue as well as thecal and granulosa cells of maturing follicles (44).

The human cell binding site may be the same for all gonococcal pili. This hypothesis is strengthened by our observation that approximately equal amounts of antigenically distinct gonococcal pili attach to human cells with the same preference for a given cell type (Fig. 4, Table I). Further, antigenically heterologous pili produce nearly equivalent inhibition of pili attachment as compared to equal weights of homologous pili (45). This suggests that the attachment portion of the gonococcal pilus that interacts with the pili binding site is equivalent for antigenically distinct pili. This preservation of the attachment moiety of the pilus for a common binding site is analogous to the reported insulin-receptor interactions for antigenically differentiable porcine, beef, or human insulin (41).

Gonococcal pili attachment is temperature sensitive. It is unlikely that this temperature dependence is the result of an energy requirement (and thus active cell pinocytosis) for attachment, as attachment, although reduced, still occurs at 4°C. Attachment of pili to fetal tonsil fibroblasts was not affected by an uncoupler of oxidative phosphorylation, DNP. An alternative explanation for the temperature dependence of attachment lies in the current understanding of cell membrane organization. The fluid-mosaic model proposed by Singer and Nicolson (46) would predict a reduced membrane fluidity at lower temperatures, possibly affecting pili binding sites partially linked to the membrane phospholipid bilayer. The binding of gonococcal pili may require a cooperation and alignment between adjacent binding sites, so that enough attachment moiety-binding site associations are made to secure the pilus. If binding site mobility is decreased, a lack of cooperation would result, decreasing the frequency of pili-binding site hits. In addition, a temperature of 37°C may also be important for the proper conformation of the pilus molecule. Previous models have been examined with respect to the temperature dependence of membrane receptor mobility. The antibody-induced Ig receptor "patch" formation phenomenon on the surface of lymphocytes is inhibited by cold (47, 48). Frye and Edidin (49), using Sendai virus-fused mouse-human heterokaryons, have elegantly demonstrated that the diffusion of surface antigens in the plane of the membrane was inhibited by lowered temperatures, whereas inhibitors of protein synthesis, ATP formation, and glutamine-dependent pathways had no effect. Similarly, rhinovirus attachment is inhibited by lower temperatures (50), and adenovirus tail fiber binding at 2°C is only 25% of that at its 37°C optimum (35). Insulin (36, 41), growth hormone (38), human chorionic gonadotropin, and luteinizing hormone (51, 52) also possess a temperature dependence for binding.

It is perhaps not surprising that we observed a pH optimum of 4.5 for gonococcal pili attachment. The pH range of urine is 4.6–8.0 with an average of 6.0 (53). Normal, midcycle cervical mucous is 7.0–8.5 (54), and vaginal secretions are acid ranging from 3.8 to 4.2 (55). Thus, gonococci isolated from infected patients possess pili that facilitate attachment optimally at the potentially hostile, acidic pH of some urogenital conditions. Although the pH of cervical mucous does not optimize attachment, it does not exclude it. Mårdh and co-workers (4, 5) have reported that a similar pH optimum (pH 4.5) exists for whole gonococcal attachment to urothelial and vaginal epithelium. Similarly, the optimum pH for adenovirus attachment (6.0–8.0 [34]), adenovirus tail fiber attachment (pH 7.0 [35]), human chorionic gonadotropin, luteinizing hormone, and insulin binding (pH 7.4 [51], 7.6 [52], and 7.5 [56], respectively) are consistent with the environment in which they interact with their respective effector cells. Another explanation of the observed enhanced attachment at pH 4.5 is that pili may aggregate at or near pH 4.5. However, even if aggregation facilitates the amount of pili bound, it is possible that this serves a functional role in gonococcal binding to human cells during gonorrhea. It is conceivable that pili-pili interactions are in part responsible for the pH 4.5 optimum for whole gonococcal attachment observed by Mårdh and co-workers (4, 5) or for the "infectious units" or clusters of gonococci frequently observed in acidic urethral exudates from patients with gonorrhea, as noted by Novotny et al. (57).

The antigenic site(s) of gonococcal pili may be removed from the attachment portion of the molecule. This is suggested by the inability to completely inhibit attachment with antibody to pili, and by the observation that antigenically different pili appear to attach to similar or identical binding sites on human cells. Homologous antibody was in general more effective than heterologous antibody to pili for blocking attachment (Table II). Homologous antibody might be expected to produce greater conformational change in the pilus structure and thus be more likely to affect the attachment moiety, due to higher affinity-avidity or greater quantities of anti-pilus antibody in the homologous pili antibody preparations. These data are consistent with other studies in which antibody to pili produced greater blockage of attachment of whole

piliated gonococci to human RBC (12), or to human buccal cells (58) when the anti-pili antibodies were prepared to pili of the piliated organisms being tested. Recently, Tramont (59) has confirmed that this same greater inhibition of piliated gonococcal attachment occurs with secretory antibody of the urogenital tract reacted with the patients infecting strain as compared to other piliated strains of gonococci. Furthermore, the secretory antibody capable of inhibiting attachment increased with gonococcal infection and decreased after treatment of gonorrhea (59). These data suggest that whereas some protection against infection might result from antibodies capable of inhibiting pili-mediated attachment, the extent of such protection might be limited by the considerable antigenic heterogeneity among pili (12, 25, 60) and the observation that blockage of pili attachment is maximal only with antibody to pili of the infecting strain (Table II; 12, 58, 59).

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