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Attachment of Gonococcal Pili to Lectin-Resistant Clones of Chinese Hamster Ovary Cells

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Pili facilitate the attachment of virulent *Neisseria gonorrhoeae* to host cells. Isolated pili and peptides from pili obtained by cyanogen bromide cleavage were used in attachment assays to Chinese hamster ovary cells and their lectin-resistant clones. Pili and the largest cyanogen bromide fragment (CNBrI) from the amino-terminal portion of the pilin molecule attached to a greater degree to the parent cell and showed 40 to 75% reduced attachment to clones deficient in cell surface oligosaccharides. The CNBrI fragment, with a molecular weight of approximately 10,000, bound specifically to host proteins with subunit molecular weights of 14,000 to 16,000 that were electrophoretically transferred onto nitrocellulose sheets from polyacrylamide gel patterns of host cells. Periodate or galactosidase treatment of pili or the CNBrI fragment markedly reduced attachment, suggesting the importance of galactose residues on pili for their attachment function. Similarly, highly purified exoglycosidase or trypsin treatment of the parent cell reduced attachment, suggesting that oligosaccharide moieties of cell surface components (glycoproteins or glycolipids or both) were receptors for pili attachment. This study indicated that the portion of the pilin molecule involved in attachment resides on the CNBrI fragment and that sugar moieties, both on pili and on the host cell, were required for optimal attachment.

The attachment of *Neisseria gonorrhoeae* to mammalian cells has been examined in a number of biological assays (3, 11, 13). Each assay indicated that more piliated organisms than non-piliated gonococci attach to a given number of mammalian cells (11, 22). Studies of the attachment of isolated pili to mammalian cells further support an attachment role for this organelle (10, 17). Attachment inhibition experiments suggest that the host receptor for pili may resemble the terminal oligosaccharide of gangliosides, namely Galβ1→3GalNAcβ1→4Galβ1→4Glc→ceramide (4).

Recently, variant cell lines selected from Chinese hamster ovary (CHO) cells were reported that had resistance to wheat germ agglutinin and ricin toxicity (2, 7). These cloned cells were shown to be deficient in the specific oligosaccharide lectin-binding sites, both in membrane glycolipids and glycoproteins. One cell line was shown to lack *N*-acetylglucosaminyltransferase, preventing the en bloc transfer of oligosaccharides needed to complete receptor synthesis.

We have studied the attachment of isolated gonococcal pili as well as the attachment of their cyanogen bromide-cleaved amino-terminal fragments (CNBrI) and carboxy-terminal fragments (CNBrII) to these CHO variant cell lines. The results suggested that sugar moieties located on both the host cell and on the CNBrI fragment from the amino-terminal portion of the pilin molecule are involved in the attachment process.

MATERIALS AND METHODS

Cells. CHO cells and two cloned cell lines, 13 and 15B, selected for their resistance to wheat germ agglutinin and ricin, respectively, were a generous gift from Stuart Kornfeld (Washington University, St. Louis, Mo.). Cells were maintained as monolayers in minimal essential medium containing 10% fetal calf serum, 2 mM L-glutamine, penicillin (50 U/ml), and 50 μg of streptomycin per ml (GIBCO Laboratories, Grand Island, N.Y.). Upon reaching 75% confluency, cells for attachment assays were harvested from flasks with a rubber policeman and suspended in phosphate-buffered saline (PBS; 0.136 M NaCl, 2.6 mM KCl, 1.4 mM KH_2PO_4, and 4.2 mM Na_2HPO_4 [pH 7.2]) containing 1% bovine serum albumin (PBSA). Cells were washed with PBSA three times by centrifugation and suspended at 25% (vol/vol) for use (17).

Enzymatic treatment of CHO cells. The parent cell line of CHO was harvested and suspended as described above. Samples of the cell suspension were incubated with one of the following combinations of enzymes: sialidase; β-galactosidase; β-*N*-acetylhexos-

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aminidase; sialidase and β-galactosidase; β-*N*-acetylhexosaminidase and β-galactosidase; sialidase, β-galactosidase, and β-*N*-acetylhexosaminidase; and trypsin. The cell suspensions were incubated with 0.01 U of the respective glycosidases (Miles Laboratories, Inc., Elkhart, Ind.) or with 100 μl of trypsin (GIBCO Laboratories; 3 mg of PBS per ml) for 1 h at 37°C with constant shaking. After being incubated, the cells were extensively washed with PBS by centrifugation and resuspended for use in the attachment assays.

Reduction and carboxymethylation of pili. Pili were isolated from *Neisseria gonorrhoeae* strain F62 as previously described (17). Pili were then reduced and carboxymethylated (5), and the modified pili were cleaved with cyanogen bromide by the method of Steers et al. (21). One hundred milligrams of cyanogen bromide was added to 100 mg of the carboxymethylated pili in 10 ml of 70% formic acid. The reaction mixture was incubated at room temperature for 20 h. At the end of the incubation, the mixture was diluted with a 10-fold excess of water and lyophilized.

Separation of CNBr fragments by gel filtration. The CNBr fragments from the carboxymethylated pili were dissolved in 10 ml of 6 M urea–0.2 M Tris-hydrochloride (pH 7.0) and loaded onto a Sephadex G-75 column (2.5 by 255 cm) (Pharmacia, Uppsala, Sweden) preequilibrated with 6 M urea–0.2 M Tris-hydrochloride (pH 7.0) and eluted with the same buffer (K. C. S. Chen and T. M. Buchanan, EMBO Workshop on Genetics and Immunobiology of Pathogenic *Neisseria*, abstr. 31, 1980). The peptide fragments in the eluant were detected by absorbance at 280 nm.

Characterization of CNBr fragments. The fractions containing similar peptides were pooled separately and desalted on a Sephadex G-25 column (2.5 by 210 cm) preequilibrated and eluted with 0.05 N NH_4OH. The desalted peptide fragments were lyophilized. Fragments were separately dissolved in 0.01 M NaOH and adjusted to pH 8.0 with 0.1 N HCl. The CNBr fragments were characterized by amino-terminal analysis, by the procedures described by Gray (8), and by carboxy-terminal analysis, using carboxypeptidase A (1).

Periodate oxidation and sodium borohydride reduction. Whole pili and the CNBrI peptide were radiolabeled with ^{125}I as described by Greenwood et al. (9), oxidized by treatment with sodium metaperiodate, and reduced by treatment with sodium borohydride to convert the resulting aldehydes to alcohols (20). Radiolabeled pili or the CNBrI peptide was suspended (up to 5 mg/ml) in 0.06 M sodium acetate (pH 4.5) containing 0.03 M sodium metaperiodate. The suspension was placed in the dark at 4°C for 28 h. After the reaction, a 10-fold molar excess of polyethylene glycol (over the sodium metaperiodate) was added. The solution was titrated to pH 8.0 with dilute NaOH, and a 25-fold molar excess of sodium borohydride in relation to the periodate was added as a 0.6 M solution in 0.6 M sodium borate buffer (pH 8.0). After 16 h at 4°C, the pH was adjusted to 5 with acetic acid, and the material was concentrated by vacuum centrifugation (Speedvac Concentrator, Savant Instruments, Inc., Hicksville, N.Y.). The concentrated radioactive material was passed over a Sephadex G-50 column (1.0 by 10.0 cm) preequilibrated with PBS (pH 7.0) and precoated with 10% bovine serum albumin. The radioactive peak solution was eluted with PBS. This material was studied for its attachment to the cells or to protein profiles from the cells on nitrocellulose sheets. Control treatment of pili and pilus fragments used sodium iodate instead of sodium metaperiodate under identical conditions.

Enzymatic degradation. Enzymatic treatment of the pili and the CNBrI peptide proceeded as follows. Approximately 150 μg of protein or peptide in 100 μl of PBS (pH 7.0) was incubated for 1 h at 37°C with 0.01 U of β-galactosidase (Miles Laboratories, Inc.) This solution was then radioiodinated as described, and the radioactive peak from a Sephadex G-50 column (1.0 by 20.0 cm) was collected. As a control, assuming no loss of enzyme by Sephadex G-50 filtration, β-galactosidase was diluted by the amount that would have occurred by labeling and diluting for use in attachment assays. The effect of this amount of enzyme on the attachment of untreated pili and untreated CNBrI fragment was then analyzed.

Attachment assay. The assay for determining the amount of radiolabeled pili and isolated peptides attached to the cells was that of Pearce and Buchanan (17). Briefly, radiolabeled protein or peptide was mixed with 200 μl of a 25% (vol/vol) suspension of cells in 1% PBSA, pH 4.5 or 7.4. This mixture was incubated with shaking at 37°C. Duplicate samples were obtained at predetermined times. Each sample was washed three times in PBSA by centrifugation. The final cell pellet was cut from the tube, and associated radioactivity was determined with a gamma counter. This radioactivity correlated with the amount of protein or peptide attached to the CHO cells.

Electrophoresis and autoradiography. Polyacrylamide gel electrophoresis followed the method of Laemmli (14). CHO cells were boiled for 5 min in 1% sodium dodecyl sulfate (SDS)–1% 2-mercaptoethanol and electrophoresed in a 12.5% polyacrylamide gel to separate the cellular components. These components were then transferred electrophoretically (Electroblot, E-C Apparatus Corp., St. Petersburg, Fla.) to a nitrocellulose sheet (24). The sheet was incubated with gentle shaking in 5% bovine serum albumin for 1 h at 37°C to block nonspecific binding, rinsed briefly in Tris-saline buffer (0.9% NaCl–10 mM Tris-hydrochloride, pH 7.4), and placed in fresh 5% bovine serum albumin containing the radioactive protein or peptide. This was incubated with shaking for 90 min at room temperature. The sheet was then washed extensively and successively with Tris-saline and Tris-saline containing 0.05% Triton X-100 (Sigma Chemical Co., St. Louis, Mo.). Finally, the air-dried sheet was placed next to Kodak X-ray film (Eastman Kodak Co., Rochester, N.Y.) in a cassette with an enhancing screen (Cronex Cassette, E. I. du Pont de Nemours & Co., Wilmington, Del.) for exposure (23). Nitrocellulose sheets, analyzed for protein patterns, were stained with 0.2% Coomassie brilliant blue–45% methanol–10% acetic acid in water and destained by using 20% methanol–2% acetic acid in water.

RESULTS

CNBr cleavage of pili. The carboxymethylated pili were cleaved with CNBr, and the resulting major fragments (CNBrI and CNBrII) were separated by a Sephadex G-75 column (Fig. 1). There were two methionines in the F62 pilus

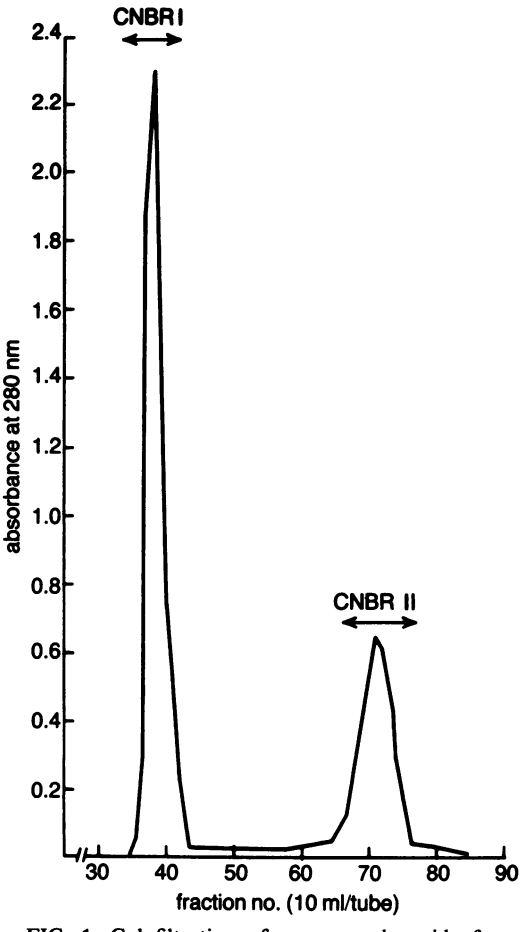

FIG. 1. Gel filtration of cyanogen bromide fragments from carboxymethylated pili (100 mg) on a Sephadex G-75 column (2.5 by 255 cm) eluted with 6 M urea–0.2 M Tris-hydrochloride (pH 7.0).

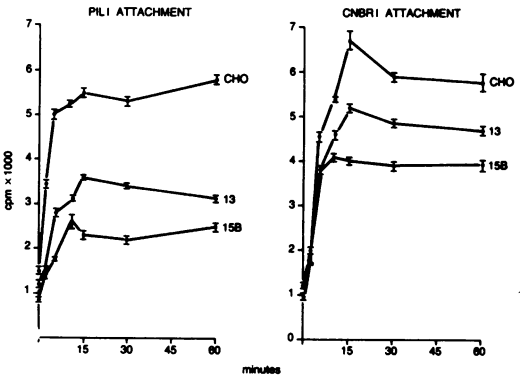

FIG. 2. Figure 2A depicts the attachment of pili at pH 4.5 to the parent CHO cell line and two of the selected clones. Figure 2B is the corresponding attachment of the CNBrI fragment. Results of attachment of whole pili and CNBrI fragments to CHO parent and mutant cells were similar at pH 7.2. Horizontal bars represent standard error.

molecule (165 total amino acids) residing at positions 7 and 102 from the amino terminus of the pilus. CNBr treatment of the pili resulted in cleavage of the methionine at position 7, with about a 20% yield; cleavage of the methionine at position 102 was approximately 90% based on amino acid analysis of the resulting fragments (K. C. S. Chen and T. M. Buchanan, manuscript in preparation). Fragment CNBrI, the largest fragment, was eluted first from the column and contained less than 10% contamination with the uncleaved pili. Based on amino acid analyses and amino acid sequence determinations for fragments CNBrI and CNBrII, CNBrI contains five tyrosines but no cystine, whereas CNBrII contains one cystine (one disulfide loop) but no tyrosine. Amino acid analyses of CNBrI fragments from two separate CNBr gel filtration preparations revealed that the contamination of CNBrII in CNBrI was about 7 and 9%, respectively, calculated from the molar ratio of tyrosine to carboxymethylcysteine in CNBrI. Fragment CNBrI was characterized as the amino-terminal portion of the pilus subunit because the major amino-terminal residue was determined to be *N*-methylphenylanine (the minor amino-terminal residue was determined to be isoleucine, presumably due to the incomplete cleavage of the methionine at position 7), and the carboxy-terminal residue was determined to be homoserine (12). Fragment CNBrII was characterized as the carboxy-terminal peptide of the pilus subunit because the amino-terminal residue was determined to be alanine, and the carboxy-terminal residue was determined to be lysine (Chen and Buchanan, manuscript in preparation). A minor fragment, CNBrIII (amino acids 1 through 7), was eluted in fractions 95 through 105 and was not used in these studies (data not shown).

Attachment to cell lines. Pili attached to all of the cell lines used, exhibiting greatest attachment to the parent CHO cell line (Fig. 2A) both at pH 4.5 and at pH 7.4. Maximum attachment occurred at approximately 15 min with each of the three cell lines. At this time, attachment to clone 13 was approximately 65% of attachment to the parent and 40% of attachment to clone 15B. Similar attachment results were obtained with the CNBrI fragment of pili (Fig. 2B), with attachment to clones 13 and 15B being 75 and 60% of that to the parent, respectively. The CNBrII fragment of pili did not attach to any of the cell lines.

Electrophoresis of proteins from the three cell lines in SDS–12.5% polyacrylamide gels showed

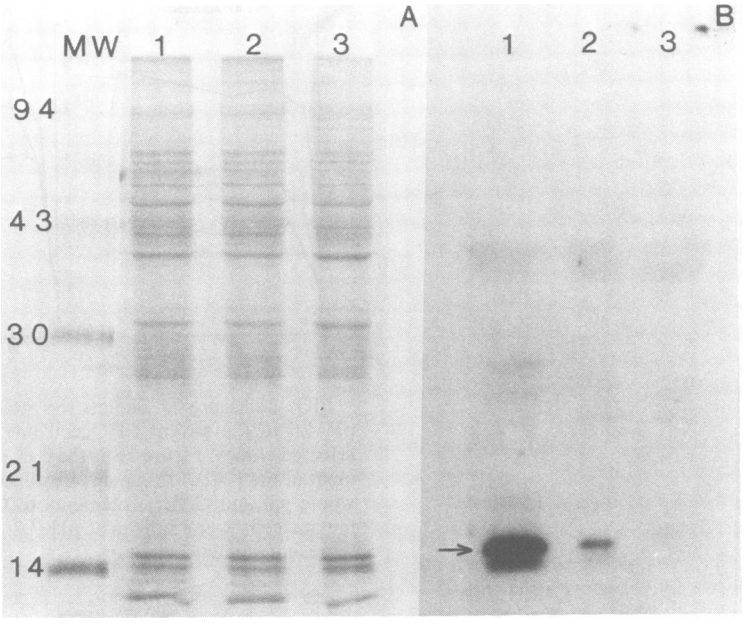

FIG. 3. Protein profile patterns of the three cell types on a 12.5% polyacrylamide gel is shown in Fig. 3A. Lane MW shows the molecular weight markers, with the numbers representing the weights $\times 10^3$. Lane 1, parent cell line; lane 2, clone 13; lane 3, clone 15B. Figure 3B is an autoradiogram of radiolabeled CNBrI attached (arrow) to these profiles, which had been electrophoretically transferred to nitrocellulose sheets.

only minor differences in the protein profiles (Fig. 3A). Autoradiography of the blotted nitrocellulose sheet which was incubated with radiolabeled pili showed nonspecific binding to the paper. However, when the radiolabeled CNBrI fragment was used, specific binding to two subunits of proteins in the low-molecular-weight region of the parent cell was observed (Fig. 3B). Only one protein subunit of clone 13 bound the CNBrI fragment, and no specific binding to clone 15B was observed. Although the autoradiogram revealed nonspecific binding of CNBrI to clone 15B, this binding did not approximate the 50% of control binding as found in the attachment assay. This discrepancy may reflect the differences in methodologies. There was no evidence of binding with the CNBrII fragment of pili (data not shown).

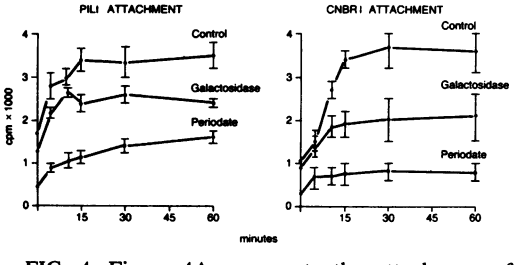

FIG. 4. Figure 4A represents the attachment of periodate- and galactosidase-treated pili to the parent cell line of CHO cells. Figure 4B shows the attachment of the identically treated CNBrI fragment to the same cells. Horizontal bars represent standard error.

These data suggest that membranes having altered surface oligosaccharides also exhibit altered attachment of pili and its CNBrI fragment. The binding site for pili on CHO cells may reside on a glycoprotein(s) with a subunit molecular weight(s) of approximately 14,000 to 16,000 as determined by SDS-polyacrylamide gel electrophoresis. Additionally, the attachment site of the pilin molecule appears to be located on the CNBrI fragment, and its functional activity is preserved despite the conditions used to obtain it.

Attachment of modified pili and CNBrI fragment. Neither the periodate-borohydride- nor the galactosidase-treated pili attached as well as untreated pili to parent CHO cells (Fig. 4A). The CNBrI peptide showed similar results (Fig. 4B). Maximal attachment for both occurred at 30 min. The attachment of periodate-treated pili was inhibited 40% as compared with the control at this time, and attachment of galactosidase-treated pili reflected 80% of control attachment. At 30 min, periodate-treated CNBrI was inhibited to 20% of control attachment, and galacto-

TABLE 1. The effect of enzymatic treatment of CHO cells on pilus attachment at pH 4.5[a]

Enzyme treatment	Attachment decrease (%)
None	0
Sialidase	20
β-Galactosidase	5
β-HexNAc	4
Sial + β-Gal	13
β-Gal + β-HexNAc	4
Sial + β-Gal + β-HexNAc	25
Trypsin	27

[a] Percent decrease shown is at 30 min. Sial, Sialidase; β-Gal, β-galactosidase; β-HexNAc, β-*N*-acetylhexosaminidase.

sidase-treated CNBrI exhibited 50% of control attachment. None of the modified pili or peptides showed any evidence of binding to blotted nitrocellulose sheets. Treatment of the pili or peptide with sodium iodate or sialidase had no effect on attachment. When β-galactosidase was added directly to the pilus-cell attachment incubation mixture in a concentration equal to its assumed level of contamination of the radiolabeled pili, there was no effect on attachment. This indicated that the effect of galactosidase treatment was due to modification of the pili or the CNBrI fragment, rather than to a modification of galactose residues on the surface of the CHO cell.

Attachment to enzymatically treated cells. Table 1 illustrates the effect of the various glycosidases and trypsin on the attachment of pili to CHO cells. Greatest inhibition was obtained with the use of trypsin or with a combination of the three glycosidases. Of the glycosidases, sialidase reduced attachment the most, whereas β-galactosidase and β-*N*-acetylhexosaminidase had minimal inhibition effects.

DISCUSSION

These studies provide additional support for a specific molecular interaction between gonococcal pili and mammalian cell surface receptors. The observation that the CNBrI fragment of gonococcal pili attaches as well as whole pili to cell surfaces suggests that the entire pilus subunit is not required for integrity of the binding site. This preservation of functional activity in CNBrI with a molecular weight of approximately 10,000 (Chen and Buchanan, unpublished data) is similar to the binding capability preserved in cholera toxin subunits with molecular weights of approximately 8,000 (16, 25). Other investigators have reported that pili contain carbohydrate, and Robertson et al. (18) have indicated that these moieties are galactose. The marked reduction in functional activity of the pilus attachment moiety, after sodium metaperiodate oxidation and sodium borohydride reduction or after galactosidase treatment, suggests that a sugar moiety, presumably nonreducing terminal galactose located on the pilus subunit, is important for the attachment function. Whether this galactose interacts specifically with mammalian cell surface proteins or contributes a "solubility" or structural configuration role, as in the hemagglutinin molecule of the influenza virus (19), remains to be determined. After periodate treatment of pili or peptide, sodium borohydride was used to reduce the resulting dialdehydes to alcohols to prevent the Schiff's base formation between the oxidized sugar(s) and proteins. Recently, Geoghegan et al. (6) have shown that periodate treatment of proteins will oxidize tyrosine residues. Oxidation of the tyrosine residues in the CNBrI fragment might contribute to a decrease in attachment. However, earlier work in this laboratory showed that pili exposed to dansyl chloride, affecting tyrosines (8), had no effect on attachment of the pili (4).

Figures 3A and 3B suggest that cellular protein molecules with subunit molecular weights of 14,000 to 16,000 contain the appropriate receptors for attachment recognition by gonococcal pili. These two proteins are presumably glycoproteins, and the portion recognized by gonococcal pili is an oligosaccharide because (i) the binding of pili and CNBrI fragments to lectin-resistant clones of CHO cells is reduced (Fig. 2A and 2B), (ii) the protein profiles from these clones resolved by SDS-polyacrylamide gel electrophoresis contain fewer of the pilus-binding proteins present on the parent cell line (Fig. 3A and 3B), and (iii) binding of pili is inhibited by treating the CHO cells with a mixture of highly purified exoglycosidases or trypsin (Table 1).

Clone 13 has *N*-acetylglucosamine at the nonreducing terminus of the oligosaccharide moiety of glycoproteins and is deficient in both sialic acid and galactose normally found on the parent cell line (2). The reduction in CNBrI and of whole pilus binding to this clone might imply a role for galactose or sialic acid (or both) in the receptor site for gonococcal pili. This inference is consistent with previous studies by Buchanan et al., indicating an influence of mammalian cell surface sialic acid on pilus attachment at pH 4.5 and an influence of cell surface galactose residues on pilus binding at pH 4.5 and 7.4 (4). Clone 15B is resistant to the plant lectin ricin and is also deficient in terminal sialic acid and galactose. It is further deficient in *N*-acetylglucosaminyl transferase activity and, consequently, may have decreased *N*-acetylglucosamine residues exposed in its oligosaccharides (15). These cells bound pili and CNBrI fragments

least well (approximately 50% of normal), a result consistent with previous studies implicating *N*-acetylhexosamine, and specifically, Galβ-1→3NAchexosamine β1→4Galβ1→4Glc→ceramide in the receptor sites for pili at neutral pH (4).

The attachment of an invasive organism to mucosal epithelium is paramount to its establishment of infection. If the mechanism of attachment can be discerned, methods to inhibit this initial step may be devised. Gonococcal pili provide a means for the virulent organism to increase the adherence of bacteria to cells. These studies suggest that the CHO parent and mutant cell lines may provide an avenue to characterize further the specific molecular interactions of pili and the cell surface.

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