PILI OF *NEISSERIA MENINGITIDIS*

Analysis of Structure and Investigation of Structural and Antigenic Relationships to Gonococcal Pili

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The recognition that attachment of pathogenic *Neisseria* to human mucosal surfaces involves specific interactions between polymeric filaments called pili and host cell receptors (1–4) has suggested new approaches to the immunoprophylaxis of meningococcal and gonococcal disease. Pili are present on meningococci and gonococci isolated from human mucosal surfaces (3, 4) and are found on meningococci after tissue invasion (5). In vitro studies indicate that pili facilitate the attachment of meningococci and gonococci to human cells and to human mucosa in organ culture (6, 7). Piliation is also correlated with resistance of gonococci to phagocytosis by human polymorphonuclear leukocytes (8). Antibodies to pili are detected after fulminant meningococcal disease (9) and in sera and mucosal secretions after natural gonococcal infection (10). These antibodies may block attachment of pathogenic *Neisseria* to human mucosal surfaces (11).

However, gonococcal pili are remarkable for their antigenic diversity (12), and different pili types may occur on meningococci (13). Antibody directed at one pilus serotype may have limited potential for blocking pilus function of heterologous serotypes. Recently (14), a parenteral pilus vaccine composed of a single pilus type was found to be ineffective in preventing gonorrhea caused by a broad range of gonococcal strains. An alternative approach to an efficacious pilus vaccine is based on the observation that gonococcal pili have structurally conserved immunorecessive regions that encompass a common receptor-binding domain, and immunodominant, structurally variable regions that determine serotype specificity (15, 16). A vaccine containing an immunogenic pilus peptide encompassing the conserved region might elicit an antibody that binds all gonococcal pili and blocks their pathogenic function (15, 16). Because pili are also the most important surface components mediating attachment of meningococci (2, 3) and because of the close genetic relationship of meningococci and gonococci (17), more detailed information was sought concerning the nature of meningococcal pili and the structural and antigenic relationships between menin-

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TABLE I

Strain Designation, Serogroup, Serotype, SDS-PAGE Type, and Source of the Isolates Used in These Studies

Meningococcal isolate	Serogroup	Serotype*	SDS-PAGE type	Source‡
3006	B	2b	I	Laboratory
P-355	B	15	XV	Laboratory
2996	B	2b	I	Laboratory
269B	B	NT	IV	CSF
1643	B	NT	I	CSF
HL-1	B	2	I	Blood
2070	C	2	IV	CSF
6083	NG	NT	II	NP (asymptomatic carrier)
3418, 3419	C	2	I	NP, CSF
RNP	B	NT	IV	NP (patient with positive blood, CSF isolates)
KB	B	8 (12, 5)	IV	CSF
RA	B	ND	IV	Blood

* NT, nontypable; ND, not done.

‡ CSF, cerebrospinal fluid; NP, nasopharynx.

gococcal and gonococcal pili. Such information may be useful in the design of a pilus vaccine effective in the prevention of both meningococcal and gonococcal disease.

Materials and Methods

Microorganisms. 13 isolates of *N. meningitidis* from 12 patients were used in these studies. The designation, serogroup, serotype, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE)[1] type and source of these isolates are listed in Table I. Isolates 3006 and P-355 (kindly supplied by Dr. W. D. Zollinger, Walter Reed Army Institute of Research, Washington, D.C.) are prototype strains for serotypes 2b and serotype 15, respectively. Before use, isolates 2996 (kindly supplied by Dr. Jan Poolman, Laboratorium Voor de Gezondheidsleer, Amsterdam) and isolates 269B, 1643, 3006, and P-355, which were originally isolated from patients with meningitis or septicemia, had been passaged multiple times in the laboratory. The remaining isolates were freshly obtained from the nasopharynx of an asymptomatic carrier, from the nasopharynx of patients with meningococcal disease, or from the blood or cerebrospinal fluid of patients with meningococcal meningitis or septicemia. The serotyping and SDS-PAGE typing of the clinical isolates was kindly performed by Dr. Carl Frasch, National Center for Drugs and Biologics, Bethesda, MD. The identification of these isolates as *N. meningitidis*, the techniques used for determining serogroup, serotype, and SDS-PAGE type, and additional characteristics of the isolates, have been previously described (2, 18). Isogenic opaque (Op) and transparent (Tr) colony variants of isolates 1643 and 3419 were also derived (19).

Neisseria gonorrhoeae isolate F62 was also used. This is a well-characterized laboratory isolate. Op and Tr derivatives of colony type 1 (T_1) and colony type 4 (T_4) of this isolate were used.

Solid media for cultivating microorganisms consisted of chocolate agar plus 1% (vol/vol) IsoVitaleX (ChocIso agar) (BBL Microbiology Systems, Cockeysville, MD) or gono-

[1] *Abbreviations used in this paper:* CNBR-1, -2, and -3, cyanogen bromide cleavage fragments 1–3; Op, opaque; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; T_1 and T_4, colony types 1 and 4; Tr, transparent; 2-ME, 2-mercaptoethanol.

coccal agar base plus 2% (vol/vol) IsoVitaleX (GcIso agar) (BBL Microbiology Systems) (2). Chocolate agar plus IsoVitaleX has previously (20) been shown to be an optimal medium for maintaining pili upon cultivation of meningococci in vitro.

Electron Microscopy. Pili were identified by negative staining electron microscopy (20). These negatively stained preparations were used to determine the electron microscopic characteristics of pili and the mean number of pili per diplococcus.

Isolation of Outer Membranes and Pili. Outer membrane preparations were obtained from meningococcal and gonococcal isolates as previously described (19). Pili were isolated using a modification of the method of Brinton et al. (21). Briefly, large agar plates were inoculated with piliated meningococcal or gonococcal colony types and incubated in a humid atmosphere with 3% CO_2 for 20 h at 37°C. Organisms were harvested from the plates with a glass rod and suspended in 0.05 M Tris-buffered saline, pH 8.0. After centrifugation at 13,000 *g* for 30 min, pili were solubilized from the pellet with 0.15 M ethanolamine (pH 10.8), subjected to overnight dialysis against 0.05 M Tris-buffered saline, pH 8.0, at 4°C, and the pilus crystals harvested by centrifugation. Purity was assessed by negative staining electron microscopy (20) and SDS-PAGE. The protein concentration of pili and outer membrane preparations was determined by the Coomassie Blue method of Bradford (22).

SDS-PAGE. Protein samples (pili or outer membrane preparations) were prepared by diluting 20 μl with sample buffer containing 0.1 M Tris-HCl (pH 6.8), 2% (wt/vol) SDS, 2% (vol/vol) 2-mercaptoethanol (2-ME), 20% (vol/vol) glycerol, and 0.001% (vol/vol) bromophenol blue. The final protein concentration of each sample was ~1.0 mg/ml. The samples were heated at 56°C for 15 min or boiled at 100°C for 3 min. In some experiments, 2-ME was omitted from the sample buffer. Protein samples were separated by SDS-PAGE on 10 or 12.5% thin-layer acrylamide gels using an LKB Multiphor electrophoresis apparatus (LKB Instruments, Inc., Gaithersburg, MD). The samples were run in duplicate on each gel, and proteins were transferred from one-half of the gel to nitrocellulose paper using the Western blot transfer technique (see below). Either Bio-Rad low molecular weight standards (Bio-Rad Laboratories, Richmond, CA) or molecular weight standards linked to a blue chromophore (prestained protein high molecular weight standards; Bethesda Research Laboratories, Bethesda, MD [BRL]) were used in duplicate on each gel. The BRL molecular weight standards were visible on nitrocellulose after Western blot transfer. After SDS-PAGE or Western blot transfer, the gel halves were fixed overnight in a solution of deionized H_2O and 50% (vol/vol) methanol, 50% (vol/vol) methanol and 12% (vol/vol) glacial acetic acid, or 50% (vol/vol) ethanol. The gels were stained with Coomassie Blue (19) or silver nitrate. Silver staining was performed using a modification of the technique of Wray et al. (23). After fixation, the gels were washed in deionized water (three times, 15 min each). In some experiments, gels were then immersed in 10% (vol/vol) gluteraldehyde in water for at least 4 h according to the technique of Schleicher and Watterson (24), and washed three additional times. Gels were bathed in 150 ml of freshly prepared staining reagent and gently agitated for 20 min. The staining reagent consisted of 0.1 N NaOH (28 ml), concentrated NH_4OH (2 ml), 20% $AgNO_3$ (wt/vol) (5 ml), and deionized H_2O (115 ml). After three 10-min washes in deionized H_2O, the gels were placed in freshly prepared developer (50 mg citric acid and 0.5 ml of 37% formaldehyde in a liter of deionized H_2O). Development was continued until the background began to turn brown, usually <20 min.

Preparation of Antibodies to Gonococcal Pilin Sequences. Pili of gonococcal strains MS-11 and R10 were purified (15). Cyanogen bromide cleavage of MS-11 and R10 gonococcal pilin proteins was performed as described by Schoolnik et al. (16) at methionine residues and resulted in three fragments, designated CNBR-1, CNBR-2, and CNBR-3. The CNBR fragments were purified by reverse-phase high pressure liquid chromatography (16). These peptides and synthetic peptide analogues of MS-11 pilin, prepared as described below, were numbered sequentially from the N-terminus. The sequence consists of 159 residues in a single polypeptide chain with two cysteines at positions 121 and 151 in disulfide linkage. CNBR-1 contains the N-terminal 7 residues; CNBR-2, residues 8–92; and CNBR-3, residues 93–159. In addition, seven peptide analogues of the MS-11 pilin

sequence were prepared by solid phase synthesis (25). They correspond to residues 21–35, 41–50, 48–60, 69–84, 107–121, 121–134, and 135–151 (see Fig. 3).

Polyclonal (rabbit) antibodies to purified whole pili, to CNBR-2 and CNBR-3, and to the seven synthetic peptides, were prepared as described (25). These antibodies were used to identify regions encoding common epitopes with meningococcal pili in the Western blot transfer technique described below.

Western Blot Transfer. Electrophoretic transfer of proteins to nitrocellulose was performed using a modification of the Western blotting technique of Burnette (26). After SDS-PAGE, the gel was apposed to a sheet of 0.45-μm-pore nitrocellulose paper (Schleicher and Schuell, Inc., Keene, NH) and placed in a "sandwich" with the following successive layers: (*a*) slab gel holder (bottom) (Miles Laboratories Inc., Elkhart, IN), (*b*) bibulous paper, (*c*) nitrocellulose paper, (*d*) gel, (*e*) bibulous paper, (*f*) two Scotch Brite pads (3M Co., St. Paul, MN), (*g*) slab gel holder (top). The sandwich was secured with plastic clamps and inserted between the electrodes of a Canalco Gel Destainer II (Miles Laboratories, Inc.). Electrophoretic transfer was accomplished in the methanol-Tris-glycine buffer system described by Towbin et al. (27) at 250 mA (LKB 2197 power supply; LKB Instruments, Inc.) for 16–22 h (overnight).

Detection of Antibody Binding to Proteins Immobilized on Nitrocellulose. After electrophoresis the nitrocellulose sheet was removed from the sandwich, placed in ~75 ml of a blocking buffer consisting of 0.5 M Tris-HCl, pH 7.5, 10% fetal bovine serum (Gibco Laboratories, Grand Island, NY), and 0.9% NaCl, and soaked for 1 h with gentle agitation on a Tektator platform (Tekpro; American Hospital Supply Corporation, Evanston, IL). After 60 min the blocking buffer was removed, and the sheet was incubated for 90 min with monoclonal antibody to meningococcal pili (kindly supplied by Dr. W. D. Zollinger, Walter Reed Army Institute of Research) or polyclonal antibody to: gonococcal pili, CNBR-2, CNBR-3, or synthetic peptide analogues of the gonococcal pilin subunit sequence (each antibody diluted 1:50 and 1:500 or, in some experiments, 1:1000 in blocking buffer). Normal mouse or rabbit and preimmune sera were used in control experiments; no reaction to meningococcal or gonococcal pilin subunits were detected with these sera. After washing in blocking buffer for 15 min, the sheet was incubated with staphylococcal protein A conjugated with peroxidase (Sigma Chemical Co., St. Louis, MO), 0.25 mg in 100 ml of blocking buffer, for 30 min. The nitrocellulose sheet was then washed twice in blocking buffer for 15 min. A 0.5% solution of 3,3′-diaminobenzidine tetrahydrochloride (Litton Bionetics Inc., Charleston, SC) in 50 ml of blocking buffer and 0.5 ml 5% H_2O_2 was prepared fresh and added after the final wash. A brown precipitate indicated antibody-protein reactions and was detected within 2–10 min.

Results

Electron Microscopic Characteristics of Meningococcal Pili. The laboratory isolates 3006, P-355, and 2996 were nonpiliated by negative-staining electron microscopy at the time of initial examination. Multiple laboratory passages of isolate 269B also resulted in loss of piliation. The remaining nine meningococcal isolates were piliated; >80% of the diplococci of each isolate contained one or more pili. The mean number of pili per diplococcus ranged from 7 to 19 for these isolates. Pili were 4–6 nm in width and extended ~500–6,000 nm in a random winding fashion from the cell surface (Fig. 1). The electron microscopic appearance and number of pili per diplococcus was similar for meningococcal isolates from different sites (e.g., NP, CSF) of the same patient, and to pili of *N. gonorrhoeae*.

Occasionally, bundles of pili, composed of 8–10 pili per bundle, were seen free or attached to diplococci (~1 bundle per 25 piliated diplococci) (Fig. 1). Unattached pili and fragments of pili were seen in all preparations. There was no

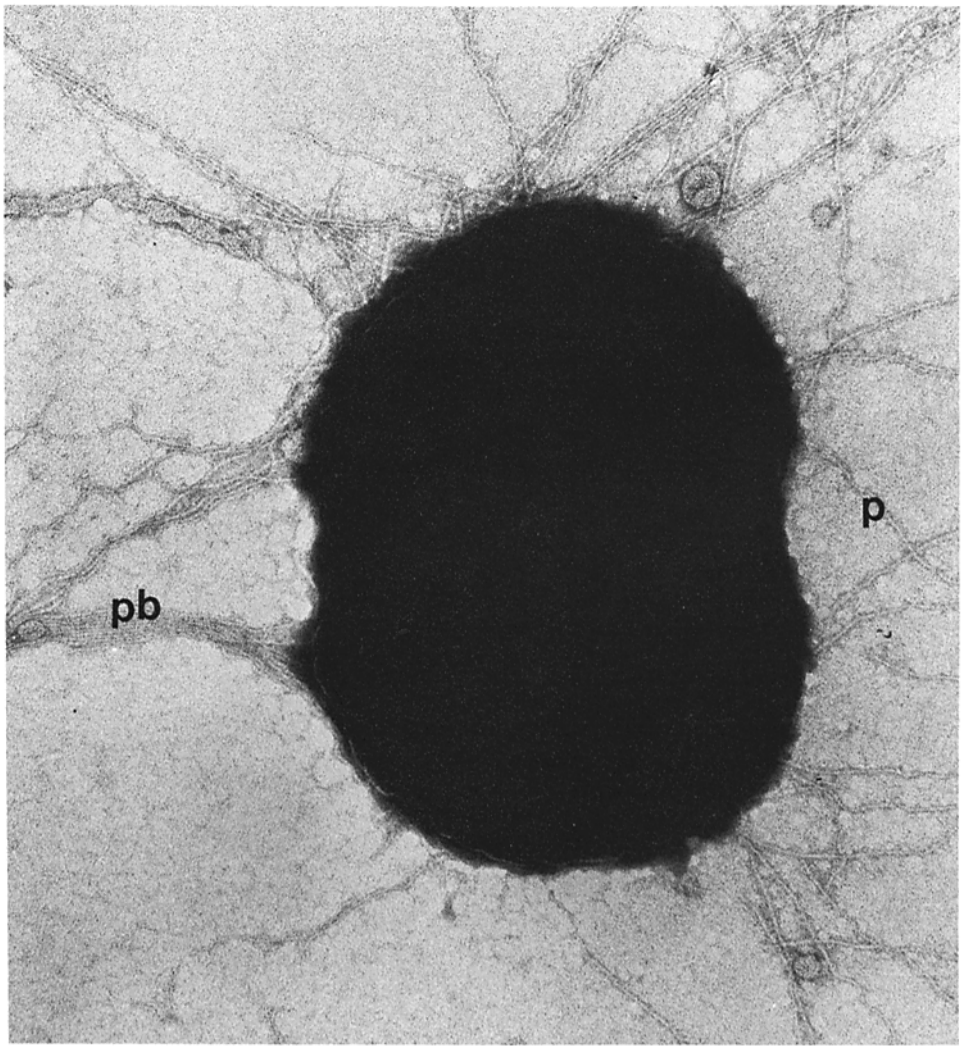

FIGURE 1. Transmission electron photomicrograph (× 99,750) of negatively stained preparation (20) of piliated meningococcal isolate 1643. Both individual pili (*p*) and pili bundles (*pb*) are present on the surface of this diplococcus.

apparent relationship between the number or distribution of pili and the serotype, SDS-PAGE type, or colony type.

Pili purified with ethanolamine solubilization and Tris-saline dialysis had a similar electron microscopic appearance to native, undetached pili, but formed large bundles composed of 20–50 pili per bundle. Although pili were the predominant structure in these preparations, outer membrane blebs were also present and were often attached to or trapped within the pili bundles. Electron-dense areas were noted between outer membrane blebs and pili bundles. Contaminating blebs were not removed by repeated solubilization and crystalization in ethanolamine and Tris-saline, respectively.

Biochemical Characteristics of Meningococcal Pili. Meningococcal pili are composed of repeating subunits (21) termed pilin. After SDS-PAGE of outer membrane or pili preparations, Coomassie Blue and silver staining procedures were used to detect meningococcal pilin. With Coomassie Blue or silver nitrate using methanol fixation, pilin stained poorly in acrylamide gels. Meningococcal pilin was best detected by silver staining after methanol/acetic acid fixation (Fig. 2*A*). The addition of a glutaraldehyde crosslinking step did not enhance detection of pilin and created excessive background staining. The location of pilin in these gels was determined by apparent subunit molecular weight (21), by reaction on Western transfer blot with a monoclonal antibody (2-1-Fc) to meningococcal pili and by reaction with antiserum to gonococcal pilin CNBR-2 fragment that crossreacted with meningococcal pilin (Fig. 2*B*).

The apparent molecular weights of pilin from different meningococcal strains vary between 17,250 and 20,600 (Table II). Pilin molecular weight did not seem to be related to serogroup, serotype, clinical syndrome or site of isolation. Pilin from meningococci that grew in opaque colonies had identical molecular weights to pilin from isogenic clones of meningococci that grew in transparent colonies (Table II). The apparent SDS-PAGE estimated pilin molecular weight was not affected by heating the pilus preparation at 56°C rather than 100°C or by the absence of 2-ME in the sample buffer (Fig. 2).

Pilin was detected in outer membrane preparations of the four meningococcal isolates that were nonpiliated by electron microscopic examination. In contrast, pilin was absent in outer membrane preparations of nonpiliated gonococci (from T4 colonies) of isolate F62. In the same outer membrane preparation of some

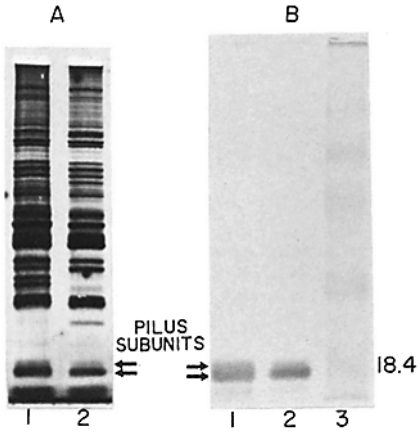

FIGURE 2. Detection of pilus subunits of meningococcal isolate 269B. (*A*) SDS-PAGE (10% gel) of outer membrane protein preparation of isolate 269B. Subunits were detected at 18,500 and 18,000 in this gel using a modification of the silver staining technique of Wray et al. (23). Subunit molecular weight was not affected by heating at 56°C (lane *1*) rather than 100°C (*2*). (*B*) Electrotransfer (Western blot transfer) of lanes *A-1* and *A-2*, respectively. These lanes were reacted with antibody to the CNBR-2 fragment of gonococcal pili. Antigen-antibody reactions were detected by peroxidase-labeled staphylococcal protein A. Both the 18,500 and 18,000 mol wt pilin bands were detected by this antibody. (Lane *3*) Molecular weight standard linked to blue chromophore allowed visibility after electrotransfer. 18.4, β-lactoglobulin (18,400 mol wt).

TABLE II

Molecular Weight of Meningococcal Pilin Subunits and Structural and Antigenic Relationships to Gonococcal Pili

Meningococcal isolate*	Pili by EM‡	Pilin molecular weight	Antibody to CNBR-2 fragment of GC pilin§	Antibody to 69–84 peptide sequence of GC pilin§
3006	−	18,125	+	ND
P-355	−	20,600	+	+
2996	−	17,250	+	−
269B	−	18,500	+	+
		18,000	+	+
1643 Op	+	19,000	+	−
1643 Tr	+	19,000	+	ND
HL-1	+	18,000	+	−
2070	+	17,500	+	+
6083	+	19,000	+	+
3418	+	17,500	+	+
3419 Op	+	17,500	+	+
3419 Tr	+	17,500	+	+
RNP	+	19,500	+	+
KB	+	18,250	+	−
RA	+	20,500	+	+
Gonococcal isolate				
F62T$_1$ Tr	+	20,000	+	+
F62T$_1$ Op	+	21,000	+	+
F62T$_4$ Tr or Op	−	—	−	−

* Op, opaque colony variant; Tr, transparent colony variant.

‡ EM, electron microscopy; +, present; −, absent.

§ GC, gonococcal; +, reactivity in Western blot of pilin with antibody; ND, not done.

meningococcal isolates, two pilin bands were detected in the 17,250–20,600 mol wt region (Fig. 2). Both of these bands reacted with antipili antibody.

Shared Antigenicity of Meningococcal and Gonococcal Pili; Implications for Sequence Homology. Antibody to CNBr fragments 2 or 3 of gonococcal pili (Fig. 3) were used to detect common antigenic determinants between meningococcal and gonococcal pili. Antibody to each of the synthetic peptide analogues of gonococcal pilin (Fig. 3) was used to deduce the location of common epitopes and regions of conserved primary structure between meningococcal and gonococcal pili.

Polyclonal antibodies to whole gonococcal pili or to the CNBR-3 fragment from gonococcal strain MS-11 bound pilin from only one meningococcal isolate, 269B. Synthetic peptides corresponding to residues 107–121, 121–134, and 135–151 of the CNBR-3 region did not react with pilin of any meningococcal isolate.

Antibody to the MS-11 CNBR-2 fragment at dilutions of 1:500 and 1:1000 reacted intensely with pilin of all meningococcal isolates (Table II, Fig. 4). Pilin was not detected by the CNBR-2 antibody in outer membrane preparations from the nonpiliated T$_4$Op or T$_4$Tr clones of gonococcal isolate F62. Antibody to CNBR-2 also did not react with outer membrane preparations of *Hemophilus*

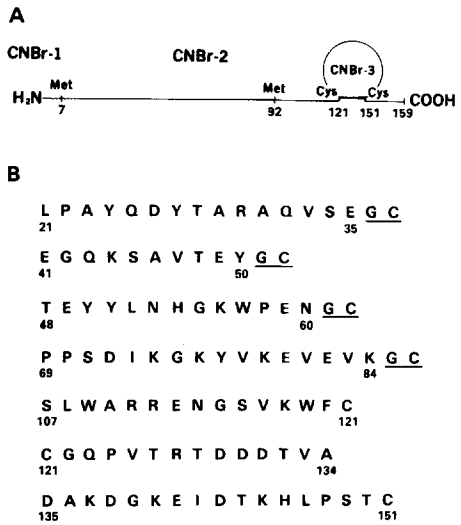

FIGURE 3. Gonococcal strain MS11 pilin CNBR fragments and synthetic peptides. Antibody to CNBR-2, CNBR-3, and the indicated synthetic peptides were used in this study with meningococcal pilins to detect common antigenic determinants specified by regions of conserved sequence. (*A*) CNBR-2 encompasses conserved receptor-binding residues and common antigenic determinants. CNBR-3 (residues 93–159) contains regions of unconserved sequence that encode strain-specific antigenic determinants (15). Synthetic peptides 21-35, 41-50, 48-60, and 69-84 correspond to regions of conserved sequence between heterologous gonococcal pili. Peptides 107-121, 121-134, and 135-151 correspond to regions of variable sequence. Underlined residues do not exist in the primary structure of gonococcal MS11 pilin; these glycine and cystine residues were added as spacers and for attachment to carrier molecules, respectively. The single-letter amino acid code is used (47).

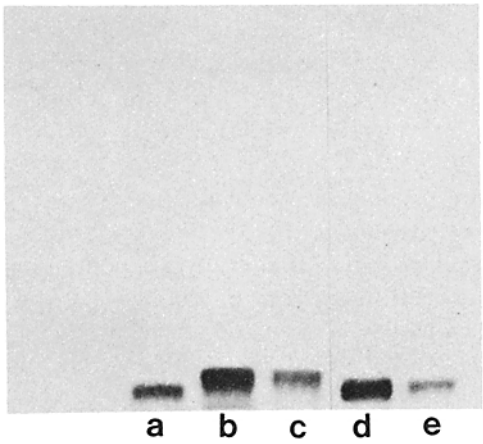

FIGURE 4. Analysis of reactivity by Western blot transfer of polyclonal antibody to the CNBR-2 of gonococcal pilus subunits with meningococcal pilus subunits. Outer membrane preparations or partially purified pili preparations from meningococcal isolates (*a*) 2070, (*b*) 1643 Op, (*c*) 1643 Tr, (*d*) 3419 Op, and (*e*) 3419 Tr were separated by SDS-PAGE (10% gel) and electroblotted to nitrocellulose. Antigen-antibody reactions were detected using peroxidase-labeled staphylococcal protein A. Antibody to CNBR-2 did not react with other meningococcal outer membrane proteins. Subunits of all 13 meningococcal isolates tested reacted with the CNBR-2 antibody.

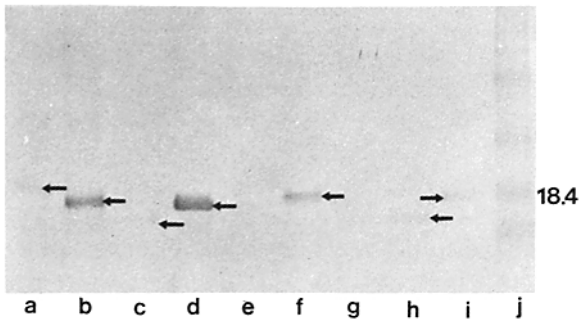

FIGURE 5. Western blot transfer analysis showing reactivity of antibody to gonococcal synthetic peptide sequence 69–84 with meningococcal pilus subunits. Outer membrane preparations or partially purified pili preparations of gonococcal isolates (*a*) F62 T_1Op, (*b*) F62T_1Tr, or meningococcal isolates (*c*) 2070, (*d*) 269B, (*e*) 1643 Tr, (*f*) P-355, (*g*) HL-1, (*h*) 3419 Op, (*i*) 6083 were separated by SDS-PAGE (12.5% gel) and electroblotted to nitrocellulose. Antibody to peptide sequence 69–84, which immunoprecipitates a receptor-binding tryptic fragment of gonococcal pili, bound (arrows) to pilus subunits of most meningococcal isolates, suggesting that a similar peptide sequence was present in most meningococcal pili.

influenzae, *Branhamella catarrhalis* or, other than pilin, any other meningococcal outer membrane protein.

Antibodies elicited by synthetic peptides 21–35, 41–50, 48–60, and 69–84, which span the CNBR-2 region, were then examined. Some meningococcal pilins were bound by a 1:50 serum dilution of antibodies to synthetic peptides 21–35 (2 of 8 isolates examined) and 48–60 (1 of 12 isolates examined). Antibody to synthetic peptide 41–50 did not react with pilins of any of eight meningococcal isolates examined. The principal CNBR-2 epitope revealed by a linear amino acid segment is encompassed by residues 69–84 (25). Antiserum to peptide 69–84 at 1:50 or 1:500 dilutions bound the pilins of most meningococcal isolates (Fig. 5, Table II), suggesting that a region of sequence homologous to this peptide exists in meningococcal pilin and specifies a common antigenic determinant.

Discussion

Infections that originate on mucus membranes entail a sequence of pathogenic events initiated by microbial adhesins and host determinants, termed receptors, to which they bind (28). Epithelial cell adherence and colonization by pathogenic microorganisms follow the exposure of a mucosal surface to an infectious inoculum. Invasion and bacteremia may ensue and result in the clinical manifestations of the infectious syndrome. Adherence, therefore, is closely linked to infectivity and appears to be a prerequisite for subsequent pathogenic events.

For many gram-negative species, adherence is conferred by filamentous adhesins termed pili, that protrude from the bacterial outer membrane (29). The morphology, primary structure, and antigenic analysis of *Escherichia coli* Gal-Gal (30), K88 (31), and K99 (32) pili, and pili prepared from gonococcal strain MS-11 (16) have been reported. The *E. coli* pili, as vaccines, have each conferred homologous protection in their respective animal model (33) or host (34, 35). Efficacy appears to be correlated with receptor-blocking, anti-pili antibodies that

presumably abrogate the adherence process. Gonococcal pilus vaccines also confer homologous protection against urethral challenge (21) but lack efficacy when tested under field conditions where antigenically heterogeneous pilus proteins are encountered (14). Recently (25), structure-function analysis of gonococcal pili has led to the preparation of synthetic peptide analogues of the pilus subunit that elicit crossreacting, receptor-blocking antibodies; these peptides were proposed as vaccine candidates. Recognizing that *N. gonorrhoeae* and *N. meningitidis* are closely related genetically (17), and with regard to certain cell-envelope antigens (36), the present study compared the electron microscopic appearance and subunit size of pili from these two species. In addition, the presence and location of common epitopes were determined, which led to the idea that common meningococcal and gonococcal pilus peptides might elicit antibodies that block mucosal adherence by members of both species.

Pili of *N. meningitidis* and *N. gonorrhoeae* were morphologically indistinguishable, and the number of pili per diplococcus was similar. Pili were noted to radiate individually from the surface of the meningococcus or as bundles of parallel aggregates (Fig. 1). Penn et al. (37) reported similar findings for gonococci and noted that changes in pilus aggregation were accompanied by changes in antigenic variation. Thus, the two forms of meningococcal pili seen in our electron microscopic studies and those recently reported by Greenblatt and Frasch (38) may represent meningococcal pili of distinct types.

The apparent subunit molecular weights of meningococcal and gonococcal pilins were also similar and varied between 17,250 and 20,600 (Table II). Although isogenic opaque and transparent variants of the same meningococcal strain produced co-migrating pilins (Table II), some strains appeared to simultaneously produce two pilins of different molecular mass (Fig. 2). These pilins probably represent two different gene products, a phenomenon also recognized in *N. gonorrhoeae* (39) and *Moraxella bovis* (G. Schoolnik, personal communication). However, this study did not exclude the possibility that the two forms were derived from incomplete posttranslational modification of a single gene product. If the identification of two pilins by SDS-PAGE and two morphologic types by electron microscopy are related, meningococci may elaborate pilus variants that are homopolymers composed of pilins of different molecular mass. These variants might also exhibit different receptor-binding specificities analogous to the pili types produced by the P-9 strain of *N. gonorrhoeae* (40).

Our data also indicate that pilin production can occur without assembly of these subunits into electron-microscopically detectable pilus filaments. Four meningococcal isolates, which had been unselectively passaged multiple times in the laboratory, were nonpiliated by electron microscopy. These isolates attached poorly to human cells (D. S. Stephens, unpublished data). However, pilin was detected in the outer-membrane preparations of each (Table II). Similar findings have recently been reported for some gonococcal isolates (41). Therefore, the expression of functional pili on the meningococcal surface appears to require factors in addition to those necessary for the production and transport of pilin to the meningococcal outer membrane. Unlike gonococcal pilus phase variation, which is correlated with chromosomal rearrangement and leads to the loss of pilin expression sites and pilin synthesis (42), loss of piliation in the meningococcus

and in some gonococcal isolates may represent a defect in the assembly of pili or the anchoring of assembled pili in the outer membrane. These findings suggest that the meningococcal pilus operon may be functionally analogous to the *E. coli* Gal-Gal pilus operon (43, 44), in which cistrons other than the pilus structural gene are required for the biosynthesis and function of normal pilus filaments.

The molecular basis for the functional and serologic properties of pili has been most extensively studied in *N. gonorrhoeae*. Pilin from gonococcal strain MS-11 consists of 159 amino acids in a single polypeptide chain with two cysteines in disulfide linkage. CNBR-2 (residues 8–92) and TC-2 (residues 31–111), a peptide prepared by arginine-specific digestion, encompass a conserved, receptor-binding region that encodes immunorecessive common antigenic determinants. CNBR-3 contains the disulfide loop in which regions of sequence variability encode immunodominant, strain-specific antigenic determinants. Synthetic peptides corresponding to seven regions of the MS-11 pilin sequence were used to identify linear antigenic determinants (25). Peptides corresponding to 41-50 and 69-84 encompass common epitopes. Peptides 121-134 and 135-151, which jointly compose the disulfide loop, encode separate MS-11 strain–specific epitopes.

In contrast, little work has been published on the molecular structure of meningococcal pili. Hermodson et al. (45) determined the meningococcal and gonococcal pilin amino acid sequences through residue 29 and found them to be highly homologous, indicating that the pili of both organisms may be derived from a common ancestral gene, a gene family now known to also include *Pseudomonas*, *Moraxella*, and *Bacteroides* species. However, no sequence data beyond residue 29 of meningococcal pilin has been reported. Consequently, antibodies to gonococcal CNBR-2, CNBR-3, and the seven synthetic gonococcal pili peptides discussed above, were used in this study to identify antigenic determinants common to gonococcal and meningococcal pili that might indicate the presence of additional homologous regions between the two proteins.

Antibodies to CNBR-3 (residues 93–159) reacted with only one meningococcal pilin, antibodies to synthetic peptides 107–121, 121–134, and 135–151 did not bind any meningococcal pilin in Western blots. This indicates that common gonococcal and meningococcal epitopes are not apparent in this region of the sequence, and, by inference, that sequence variability may be considerable. In contrast, gonococcal CNBR-2 bound all tested piliated meningococcal strains, indicating that common antigenic determinants exist in regions of conserved sequence. Their identities were sought with antibodies to the four synthetic peptides that correspond to segments of the gonococcal CNBR-2 sequence. Some shared antigenicity was noted between meningococcal pilin and peptides 21-35 and 48-60, suggesting that sequence homology exists between gonococcal pilin and some meningococcal pilins in these regions. Antibody to peptide 69-84, which bound all tested gonococcal pilins (25), also bound most meningococcal pilins, indicating that a highly conserved segment in this region encodes an antigenic determinant common to both proteins. Anti–69-84 also blocks the attachment of heterologous gonococci to endometrial cells (46), a finding that suggests that this site is within or contiguous to the receptor-binding domain. Therefore, it is conceivable that the location and structure of this domain is the same for meningococcal and gonococcal pili.

This study has provided additional evidence that gonococcal and meningococcal pili are morphologically and chemically related and that they belong to a single serologic family of crossreacting antigens. In addition, the existence of structurally conserved regions within or adjacent to their respective receptor-binding domains suggests that common peptides might form the basis for a new vaccine for the prevention of both meningococcal and gonococcal infections.

Summary

To provide information useful for the design of a pilus vaccine effective for the prevention of both meningococcal and gonococcal disease, the electron microscopic morphology of meningococcal pili and the structural and antigenic relationships of meningococcal pili to gonococcal pili were investigated. Meningococcal pili were 4–6 nm in width, extended 500–6,000 nm from the organism surface, and occurred singly or in bundles composed of 8–10 pili per bundle. Meningococcal pilin varied between 17,250 and 20,600 daltons. Pilin was present in outer membrane preparations of some meningococcal isolates that were nonpiliated by electron microscopic examination.

Antibodies to gonococcal pili, cyanogen bromide cleavage fragments of gonococcal pilin, or synthetic peptide analogues corresponding to regions of the gonococcal pilin sequence, were used to detect common meningococcal and gonococcal antigenic determinants that might indicate the existence of a conserved sequence beyond residue 29. Antibody to intact gonococcal pili or to the variable CNBR-3 region of gonococcal pilin detected little shared antigenicity with meningococcal pilin. However, pilin from all tested meningococcal isolates reacted with antibody to the CNBR-2 fragment of gonococcal pilin, a region highly conserved among gonococcal strains. Meningococcal pilins were also broadly crossreactive with antibody to a synthetic peptide corresponding to residues 69–84 of the gonococcal sequence, a part of the CNBR-2 region that appears to be critical for gonococcal receptor-binding function. If a sequence similar to 69–84 is also important for receptor-binding function in meningococcal pili, a peptide corresponding to this region may elicit antibodies that block the adherence function of pili elaborated by both *Neisseria gonorrhoeae* and *N. meningitidis*.

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References

1. Pearce, W. A., T. M. Buchanan. 1978. Attachment role of gonococcal pili. Optimum conditions and quantitation of adherence of isolated pili to human cells in vitro. *J. Clin. Invest.* 61:931.
2. Stephens, D. S., and Z. A. McGee. 1981. Attachment of *Neisseria meningitidis* to human mucosal surfaces: influence of pili and type of receptor cell. *J. Infect. Dis.* 143:525.
3. Craven, D. E., M. S. Peppler, C. E. Frasch, L. F. Mocca, P. P. McGrath, and G.

Washington. 1980. Adherence of isolates of *Neisseria meningitidis* from patients and carriers to human buccal epithelial cells. *J. Infect. Dis.* 142:556.

4. James, J. F., and J. Swanson. 1978. Piliation of gonococci in vivo. *J. Infect. Dis.* 137:94.
5. Stephens, D. S., K. M. Edwards, F. Morris, and Z. A. McGee. 1982. Pili and outer membrane appendages on *Neisseria meningitidis* in the cerebrospinal fluid of an infant. *J. Infect. Dis.* 146:568.
6. Stephens, D. S., L. H. Hoffman, and Z. A. McGee. 1983. Selectivity for human nasopharyngeal mucosa of piliated *Neisseria meningitidis*. *Med. Trop.* 43(1):73.
7. McGee, Z. A., A. P. Johnson, and D. Taylor-Robinson. 1981. Pathogenic mechanisms of *Neisseria gonorrhoeae*: observations on damage to human fallopian tubes in organ culture by gonococci of colony type 1 or type 4. *J. Infect. Dis.* 143:413.
8. Dilworth, J. A., J. O. Hendley, and G. L. Mandell. 1975. Attachment and ingestion of gonococci by human neutrophils. *Infect. Immun.* 11:512.
9. Poolman, J. T., C. T. P. Hopman, and H. C. Zanen. 1983. Immunogenicity of meningococcal antigens as detected in patient sera. *Infect. Immun.* 40:398.
10. Tramont, E. C., and T. Ciak. 1978. Antigonococcal antibodies in genital secretions. *In* Immunobiology of *Neisseria gonorrhoeae*. Brooks, Gotschlich, Holmes, Sawyer, and Young, editors. American Society for Microbiology, Washington, D.C. 274–278.
11. McChesney, D., E. C. Tramont, J. W. Boslego, J. Ciak, J. Sadoff, and C. C. Brinton. 1982. Genital antibody response to a parenteral gonococcal pilus vaccine. *Infect. Immun.* 36:1006.
12. Buchanan, T. M. 1975. Antigenic heterogeneity of gonococcal pili. *J. Exp. Med.* 141:1470.
13. Trust, T. J., R. M. Gillespie, A. R. Bhatti, and L. A. White. 1983. Differences in the adhesive properties of *Neisseria meningitidis* for human buccal epithelial cells and erythrocytes. *Infect. Immun.* 41:106.
14. Boslego, J., R. Chung, J. Sadoff, D. McChensney, M. Piziak, J. Ciak, J. Brown, W. Caldwell, D. Berliner, G. Seitter, C. Brinton, and E. Tramont. 1984. Efficacy trail of a purified gonococcal pilus vaccine. *24th Interscience Conf. Antimicrobial Agents Chemother., Wash. D.C.* Abstr. 294. p. 142.
15. Schoolnik, G. K., J. Y. Tai, and E. C. Gotschlich. 1983. A pilus peptide vaccine for the prevention of gonorrhoeae. *Prog. Allergy.* 33:314.
16. Schoolnik, G. K., R. Fernandez, J. Y. Tai, J. Rothbard, and E. C. Gotschlich. 1984. Gonococcal pili: primary structure and receptor-binding domain. *J. Exp. Med.* 159:1351.
17. Kingsburg, D. T. 1967. Deoxyribonucleic acid homologies among species of the genus *Neisseria*. *J. Bacteriol.* 94:870.
18. Frasch, C. E. 1979. Noncapsular surface antigens of *Neisseria meningitidis*. *Semin. Infect. Dis.* 2:304.
19. Stephens, D. S., and Z. A. McGee. 1983. Association of virulence of *Neisseria meningitidis* with transparent colony type and low-molecular-weight outer membrane proteins. *J. Infect. Dis.* 147:282.
20. McGee, Z. A., C. H. Street, C. L. Chappel, E. S. Cousar, F. Morris, and R. G. Horn. 1979. Pili of *Neisseria meningitidis*: effect of media on maintenance of piliation, characteristics of pili and colonial morphology. *Infect. Immun.* 24:194.
21. Brinton, C. C., J. Bryan, J. A. Dillon, N. Guerina, L. J. Jacobson, S. Kraus, A. Labik, S. Lee, A. Levene, S. Lim, J. McMichael, S. Polen, K. Rogers, A. C. C. To, and S. C. M. To. 1978. Uses of pili in gonorrhea control: role of pili in disease, purification and properties of gonococcal pili and progress in the development of a gonococcal pilus vaccine for gonorrhoea. *In* Immunobiology of *Neisseria gonorrhoeae*. Brooks,

Gotschlich, Holmes, Sawyer, Young, editors. American Society for Microbiology, Washington, D.C. 155–178.

22. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248.
23. Wray, W., T. Boulikas, V. P. Wray, and R. Hancock. 1981. Silver staining of proteins in polyacrylamide gels. *Anal. Biochem.* 118:197.
24. Schleicher, M., and D. M. Watterson. 1983. Analysis of differences between Coomassie Blue stain and silver stain procedures in polyacrylamide gels: conditions for the detection of calmodulin and troponin C. *Anal. Biochem.* 131:312.
25. Rothbard, J. B., R. Fernandez, and G. K. Schoolnik. 1984. Strain-specific and common epitopes of gonococcal pili. *J. Exp. Med.* 160:208.
26. Burnette, W. N. 1981. Electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal. Biochem.* 112:195.
27. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350.
28. Beachey, E. H. Bacterial adherence: adhesin-receptor interactions mediating the attachment of bacteria to mucosal surfaces. *J. Infect. Dis.* 143:325.
29. Brinton, C. 1965. The structure, function, synthesis and genetic control of bacterial pili and a molecular model for DNA and RNA transport in gram-negative bacteria. *Trans. NY Acad. Sci.* 27:1003.
30. O'Hanley, P., et al., D. Lark, S. Normark, S. Falkow, and G. Schoolnik. 1983. Mannose-sensitive and Gal-Gal binding *E. coli* pili from recombinant strains. *J. Exp. Med.* 158:1713.
31. Klemm, P. 1981. The complete amino acid sequence of the K88 antigen, a fimbrial protein from *Escherichia coli. Eur. J. Biochem.* 117:617.
32. de Graaf, F. K., P. Klemm, and W. Gaastra. 1981. Purification, characterization and partial covalent structure of the adhesive antigen K99 of *Escherichia coli. Infect. Immun.* 33:877.
33. O'Hanley, P., D. Lark, S. Falkow, and G. Schoolnik. 1985. Molecular basis of *Escherichia coli* colonization of the upper urinary tract in BALB/c mice: Gal-Gal pili immunization prevents *E. coli* pyelonephritis in the BALB/c mouse model of human pyelonephritis. *J. Clin. Invest.* 75:347.
34. Ruther, J. M., and G. W. Jones. 1973. Protection against enteric disease caused by *Escherichia coli*: a model for vaccination with a virulence determinant? *Nature (Lond.).* 242:531.
35. Isaacson, R. E., E. A. Dean, R. L. Morgan, and H. W. Moon. 1980. Immunization of suckling pigs against enterotoxigenic *Escherichia coli*-induced diarrheal disease by vaccinating dams with purified K99 or 987P pili: antibody production in response to vaccination. *Infect. Immun.* 29:824.
36. Tramont, E. C., J. C. Sadoff, and M. S. Artenstein. 1974. Cross-reactivity of *Neisseria gonorrhoeae* and *Neisseria meningitidis* and the nature of antigens involved in the bactericidal reaction. *J. Infect. Dis.* 130:340.
37. Penn, C. W., N. J. Parsons, D. R. Veale, and H. Smith. 1980. Antigenic heterogeneity associated with pilus aggregation and autoagglutinability in *Neisseria gonorrhoeae. J. Gen. Microbiol.* 121:195.
38. Greenblatt, J. J., and C. E. Frasch. 1984. Differences in morphology and binding properties of pili from disease and carrier isolates of *Neisseria meningitidis. 24th Interscience Conf. Antimicrobial Agents Chemother., Wash. DC.* Abstr. 689. p. 209.

39. Salit, I. E., M. Blake, and E. C. Gotschlich. 1980. Intra-strain heterogeneity of gonococcal pili is related to opacity colony variance. *J. Exp. Med.* 151:716.
40. Lambden, P. R., J. N. Robertson, and P. J. Watt. 1981. The preparation and properties of alpha and beta pili from variants of *Neisseria gonorrhoeae* P-9 *J. Gen. Microbiol.* 124:109.
41. Swanson, J. 1984. Gonococcal variants with P-colony morphologies. *4th Int. Pathogenic Neisseria Conf., Asilomar, CA.* Abstr. 80.
42. Meyer, T. F., N. Mlawer, and M. So. 1982. Pilus expression in *Neisseria gonorrhoeae* involves chromosomal rearrangement. *Cell.* 30:45.
43. Normark, S., D. Lark, R. Hull, M. Norgren, M. Baga, P. O'Hanley, G. Schoolnik, and S. Falkow. 1983. Genetics of digalactoside-binding adhesin from a uropathogenic *Escherichia coli* strain. *Infect. Immun.* 41:942.
44. Mooi, F. R., N. Harns, D. Bakker, and F. K. de Graff. 1981. Organization and expression of genes involved in the production of the K88ab antigen. *Infect. Immun.* 32:1155.
45. Hermodson, M. A., K. C. Chen, and T. M. Buchanan. 1978. Neisseria pili proteins: amino-terminal amino acid sequences and identification of an unusual amino acid. *Biochemistry.* 17:442.
46. Rothbard, J. B., R. Fernandez, L. Wang, N. N. G. Teng, and G. K. Schoolnik. 1985. Antibodies to peptides corresponding to a conserved sequence of gonococcal pilins block bacterial adhesion. *Proc. Natl. Acad. Sci. USA.* 82:915.
47. IUPAC-IUB. Commission on Biochemical Nomenclatures. 1968. A one-letter notation for amino acid sequences, tentative rules. *J. Biol. Chem.* 143:3557.