STRAIN-SPECIFIC AND COMMON EPITOPES OF GONOCOCCAL PILI

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Pili are polymeric filaments extending from the cell envelope of *Neisseria gonorrhoeae* that promote infectivity by binding to epithelial cell surfaces (1–6). Since antisera directed at pili may block mucosal adherence by gonococci and therefore their capacity to colonize urogenital epithelium, pili have been proposed as vaccine constituents for the prevention of gonorrhea (1). However, gonococcal pili are antigenically heterogeneous and the protection conferred by a vaccine composed of undenatured pilus filaments from a single gonococcal strain appears to be strain specific. Yet, antisera against pili from one strain can cross-react weakly with pili from other strains, implying that a common determinant could exist (1). Characterization of a common determinant might lead to the development of a suitable immunogen for the prevention of gonorrhea.

To investigate the structural basis of these serologic properties, we recently have determined the complete amino acid sequence of pilin isolated from the transparent colonial variant (Tr)[1] of strain MS11 and the partial sequence of R10 (Tr) pilin (7). The comparison of these two sequences and the previously reported serological properties of antisera directed against the two principal cyanogen bromide–generated fragments of pilin (CNBr-2, residues 9–92 and CNBr-3, residues 93–159) have enabled us to construct an antigenic model of the protein (8). The immunodominant, antigenically variable region is within CNBr-3, while CNBr-2 contains a conserved receptor binding region and possibly an immunorecessive epitope common to all gonococcal pilin (8).

In this study, peptides corresponding to both variable and constant regions of MS11 pilin were synthesized in order to (*a*) more precisely determine the antigenic structure of pilin, (*b*) understand the molecular details of the antigenic variation in the protein, and (*c*) identify regions of the protein that might elicit antisera that would inhibit the binding of all gonococcal strains to eucaryotic cells. Each peptide was tested for its ability to be bound by polyclonal sera

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[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; CNBr-2, cyanogen bromide–generated fragment of pilin, residues 9–92; CNBr-3, CNBr-generated fragment of pilin, residues 93–159; DCC, dicyclohexylcarbodiimide; SMCC, succinimidyl 4-(*N*-maleimido-methyl)cyclohexane-1-carboxylate; Tr, transparent colonial variant.

directed against pili isolated from homologous and heterologous gonococcal strains. In addition, antisera directed against CNBr-2 and CNBr-3 were tested for their ability to bind the peptides. An antigenic determinant common to all strains of pili and two separate strain-specific epitopes were defined. We also have demonstrated that antisera against CNBr-2 recognizes a different common determinant than that seen by sera against the intact pilus filaments.

Materials and Methods

Purification of Pili and the Generation of Cyanogen Bromide Fragments. Pili purification and the generation and isolation of the cyanogen bromide–generated fragments, CNBr-2 and CNBr-3, from MS11(Tr) and R10(Tr) strains of *N. gonorrhoeae* were performed as described previously (7).

Specific Anti-Pili Antisera. Antisera against undenatured pili from MS11(Tr), R10(Tr), and against their two major cyanogen bromide–generated fragments (CNBr-2 and CNBr-3) were prepared in female New Zealand White rabbits. 50 μg of each protein was emulsified in 1 ml of phosphate-buffered saline with an equal volume of complete Freund's adjuvant and administered by multiple subcutaneous injections. Booster injections in Freund's incomplete adjuvant were administered 6 wk later. 10 d thereafter, the animals were bled by cardiac puncture and the sera were filter sterilized and stored at 4°C.

Selection of the Peptides Synthesized. Peptides believed to contain strain-specific epitopes were chosen according to differences in sequence between MS11 and R10 pilin. All known sequence differences between these two proteins occur between residues 93 and the carboxyl terminus, residue 159 (7, 8). Particular importance was given to regions where amino acid substitutions significantly affected either the charge, the hydrophobicity/hydrophilicity, or the predicted secondary structure of the region. Regions of sequence homology between residues 8 and 92 were synthesized in an attempt to define a common epitope. We avoided regions that were very hydrophobic, but did not simply choose areas of great hydrophilicity. The association of the hydrophilicity of a region with antigenicity neglects the importance of hydrophobic interactions when an antibody binds an antigen (9). Consequently, regions that had both hydrophobic and hydrophilic character were preferred.

The possible conformations that the peptide can adopt when conjugated to a carrier protein also were considered. Only if a peptide adopts the identical secondary structure that it has in the intact protein will it both bind sera generated against the intact protein and elicit antibodies that cross-react with that protein. Of the three common secondary structures present in proteins, β-pleated sheets, α-helices, and reverse or β-turns, we believe reverse turns have the highest probability of being present in an isolated oligopeptide <15 residues in length. Therefore, a peptide containing a region with high turn probability will be more likely to be identical to the corresponding region in the intact protein than regions predicted to be sheets or helices.

The β-turns were emphasized further by conjugating the peptide in an orientation that resulted in the β-turn being distal from the linkage to the carrier protein.

Synthesis and Characterization of Peptides. Peptides were synthesized by solid phase techniques (10) on a Beckman model 990B peptide synthesizer using commercially available amino acid polystyrene resins and t-Boc-protected amino acids (Peninsula Laboratories, Belmont, CA), with the following side chain protecting groups: O-benzyl esters for Asp, Glu, Thr, and Ser; tosyl for Arg and His; para-methoxybenzyl for Cys, orthochlorobenzyloxycarbonyl for Lys, and 2,6-dichlorobenzyl for Tyr. All couplings were performed using a 2.5 molar excess of t-Boc amino acid and dicyclohexylcarbodiimide (DCC) over the number of milliequivalents of amino acid on the resin. In the case of Asn and Gln, a 2.5 molar excess of the amino acid, DCC, and *N*-hydroxy triazole was used.

All couplings were >99% complete, as determined by the reaction of the resin with ninhydrin (11). The peptides were deprotected and removed from the resin simultaneously by treatment with anhydrous hydrogen fluoride in the presence of anisole, dimethyl sulfide, and indole. The peptides were separated from the various organic side products

by extraction with ether and isolated from the resin by extraction with 5% acetic acid and subsequent lyophilization. The purity of the crude product was determined by high pressure liquid chromatography on a C-18 reverse phase column (Merck, Darmstadt, Germany) and by amino acid analysis. The peptides used in these experiments were not further purified since they all contained >90% of the desired product.

Conjugation of the Peptides to Carrier Proteins. The peptides were conjugated to bovine serum albumin (BSA) using succinimidyl 4-(*N*-maleimido-methyl)cyclohexane-1-carboxylate (SMCC) (Pierce, Rockford, IL) as described (12). Briefly, 10 mg of the carrier protein were dissolved in 2 ml phosphate-buffered saline, pH 7.4, and combined with 5 mg of crosslinker dissolved in 500 μl of dimethylformamide. After an hour, the conjugate was separated from unreacted crosslinker by gel filtration on a G-25 column in 0.1 M phosphate, pH 6.0. The peptide to be conjugated was dissolved in 0.1 M borate, pH 9.1 and reduced with $NaBH_4$ (100 μl of 0.1 M stock). After 5 min the pH of the borate solution was lowered to 1 with 1 M HCl, to remove the excess $NaBH_4$, and then raised to pH 6 with 1 M NaOH and combined with the carrier-crosslinker conjugate. After incubating for an additional hour the carrier-peptide conjugate was desalted on a G-25 column in 0.1 M NH_4HCO_3. The degree of conjugation was quantitated by comparing the amino acid composition of the carrier protein before and after reaction with the peptide. The conjugates used in this study all contained between 15 and 25 peptides per molecule of BSA.

Determination of the Cross-reactivity of Sera Against Intact Pili and CNBr-2 and CNBr-3 of Pilin. A two-step competitive ELISA was used to determine the cross-reactivity of sera directed against MS11 and R10 pili and their cyanogen bromide–generated fragments, CNBr-2 and CNBr-3 (13). Briefly, each antisera was diluted to a concentration that resulted in 60% maximal binding to the antigen on a solid phase. The sera (100 μl) containing increasing concentrations of either MS11 or R10 pili were added to wells precoated with 0.1 μg of either MS11 or R10 pili. The plates were incubated for 3 h at 37°C, washed, treated with alkaline phosphatase–conjugated goat anti-rabbit IgG (Cappel, West Chester, PA), washed, treated with *p*-nitrophenyl phosphate (Sigma, St. Louis, MO), and read at 405 nm.

Binding of Polyclonal Sera to Peptide-Protein Conjugates. Rabbit polyclonal antibodies against either whole pili or cyanogen bromide–generated fragments of pilin, CNBr-2 and CNBr-3, were assayed for their ability to bind peptide-BSA conjugates by using solid phase binding assays. Briefly, 96-well plates were coated with 10 μg of the peptide-BSA conjugate, washed, treated with serially diluted antisera, washed, and treated either with ^{125}I protein A (Amersham, Arlington Heights, IL) or a goat anti-rabbit alkaline phosphatase conjugate (Cappel, West Chester, PA) followed by *p*-nitrophenyl phosphate (Sigma, St. Louis, MO). The radioactive wells were cut from the plate and counted, while the wells containing the antibody-enzyme conjugates were read at 405 nm.

Absorption of Antisera with Peptide-Carrier-Sepharose Conjugates. The peptide-BSA conjugates were reacted with cyanogen bromide–activated Sepharose (Pharmacia, Piscataway, NJ) as described (14). Anti-R10 and anti-MS11 pilus sera (1 ml) were absorbed with 100 μl of either peptide 121-134-BSA or 135-151-BSA sepharose for 2 h at room temperature. The mixture was spun and the absorbed sera was separated and assayed for the complete removal of the specific anti-peptide antibody by a solid phase binding assay.

Results

Shared Antigenicity of R10 and MS11 Pili. Previous studies by Brinton et al. (1) demonstrated that antisera against gonococcal pili are directed principally at strain-specific epitopes. However, the sera also contain antibodies that cross-react with pili of other strains. This cross-reactivity could be due to either differential binding at strain-specific epitopes as a result of amino acid sequence variations or due to the presence of a weakly immunogenic determinant common to all gonococcal pili. Polyclonal sera against the R10 and MS11 pili used in this study

initially were tested using a two-step inhibition solid phase binding assay to determine their specificity (13). As seen in Fig. 1,*A* and *B*, the anti-whole pili sera is quite strain specific. The shared antigenicity detected by both sera is only ~5%. Whether the cross-reactivity of the sera is due to epitopes common to all pili or due to differential binding at strain-specific determinants was partly answered by examining the antigenic relatedness detected by the sera against two cyanogen bromide–generated fragments of pilin, CNBr-2, residues 8–92 and CNBr-3, residues 93–159. A marked difference is apparent in the shared antigenicity detected by the sera directed against CNBr-2 (Fig. 1,*C* and *D*) from that against CNBr-3 (Fig. 1,*E* and *F*). In the former case the cross-reactivity was almost complete, whereas antisera against the carboxyl terminal fragment displayed high strain specificity. These experiments indicate that a weakly immu-

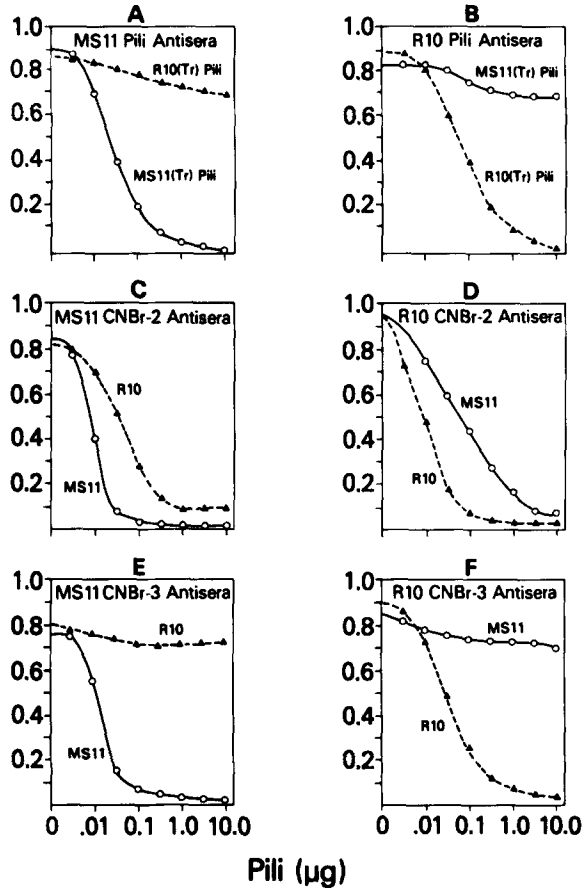

FIGURE 1. The specificity of antisera generated against either intact MS11 pili, intact R10 pili, or each of their principal cyanogen bromide–generated fragments, CNBr-2 (residues 9–92) and CNBr-3 (residues 93–159). Antisera against intact MS11 pili (*A*), MS11 (CNBr-2 (*C*), and MS11 CNBr-3 (*E*) were assayed in a two-step competitive ELISA for their ability to bind wells coated with MS11 (Tr) pili in the presence of increasing amounts of either soluble R10 or MS11 pili (abscissa). Similarly, antisera against intact R10 pili (*B*), R10 CNBr-2 (*D*), and R10 CNBr-3 (*F*) were tested for their ability to bind wells coated with R10 (Tr) pili in the presence of increasing concentrations of soluble MS11 and R10 pili (abscissa).

nogenic, common epitope(s) exists between residues 9–92 and that the strain-specific, immunodominant determinant(s) is located between residues 93–159. The precise location of each of these epitopes was determined by examining the antigenicity of synthetic peptides corresponding to regions of the MS11 sequence.

Location of Strain-specific Antigenic Determinants. To locate more precisely the strain-specific epitopes within the carboxyl terminal cyanogen bromide–generated fragment of gonococcal pilin, four peptides were synthesized corresponding to residues 95–107, 107–121, 121–134, and 135–151 (Table I). Peptides 121–134 and 135–151 compose a disulfide loop, while 95–107 and 107–121 correspond to the region on the amino terminal side of this loop. The reduced sulfhydryl of the cysteine at either the carboxyl or amino terminus of each peptide was used to conjugate it to BSA using the hetero-bifunctional crosslinker SMCC. Microtiter plates were coated with the BSA-peptide conjugates and antisera against MS11 and R10 pili were tested for their ability to bind to them.

The homologous anti-MS11 serum binds the two peptides corresponding to the disulfide loop, but not the peptides outside this region (Fig. 2*A*). Anti-R10 serum does not bind all four of these conjugates (Fig. 2*C*). Additional proof that these peptides contain an epitope specific for MS11 pili is provided by binding experiments with antisera against the carboxyl terminal cyanogen bromide–generated fragments (CNBr-3) or MS11 and R10. Anti-MS11 CNBr-3 serum

TABLE I

Amino Acid Sequences of the Eight Synthetic Oligopeptides and Their Corresponding Location in MS11 Pilin

L P A Y Q D Y T A R A Q V S E G C*	
21	35 ——
E G Q K S A V T E Y G C	
41	50 ——
T E Y Y L N H G K W P E N G C	
48	60 ——
P P S D I K G K Y V K E V E V K G C	
69	84 ——
C S G V N N E I K G K K L S	
—	107
95	
S L W A R R E N G S V K W F C	
107	121
C G Q P V T R T D D D T V A	
121	134
D A K D G K E I D T K H L P S T C	
135	151

* Underlined residues do not exist in the primary structure of MS11 pilin. Additional glycine and cysteine residues were added as spacers and as a mode of attachment to carrier molecules, respectively.

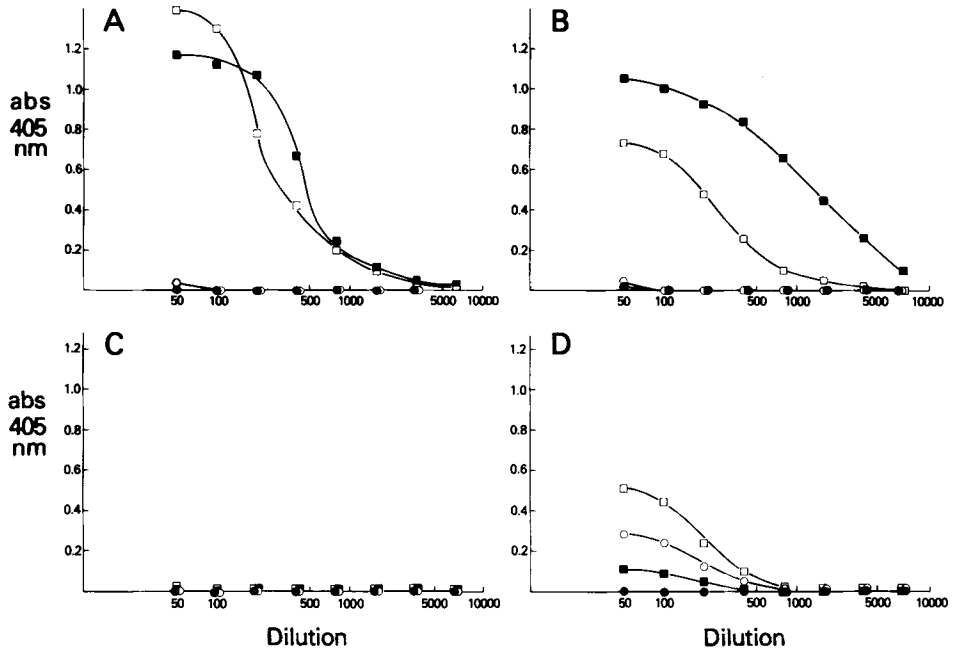

FIGURE 2. Reaction of anti-gonococcal pili sera with peptide-BSA conjugates corresponding to regions of sequence variation between strains. Anti-MS11 pili (*A*), anti-MS11 CNBr-3 (*B*), anti-R10 pili (*C*), and anti-R10 CNBr-3 (*D*) antisera were assayed for their ability to bind peptides corresponding to residues 95–107 (●), 107–121 (○), 121–134 (■), and 135–151 (□) of the MS11 pilin sequence. The enzyme-linked immuno-binding assays were performed as described in Materials and Methods. Each point represents the average of three determinations.

bound the two peptides comprising the disulfide loop even at dilutions as high as 1:6,400 (Fig. 2*B*), whereas antiserum to the same fragment from R10 pilin binds very weakly and only at high serum concentrations (Fig. 2*D*).

The observation that the two peptides that are bound both by polyclonal sera against whole MS11 pili and against CNBr-3 are juxtaposed in sequence, creates the possibility that either there is a single epitope whose component amino acids are shared by the two peptides or that there are two independent epitopes, one within each peptide. This ambiguity was resolved easily by absorbing the MS11 pilus antiserum with one peptide conjugate and then studying the absorbed serum's ability to bind the second peptide conjugate. If there is a single shared determinant, then the absorption should reduce the amount of antibodies binding to the second peptide. If there are two epitopes, the absorption should not affect the serum's ability to bind the other conjugate. Absorption with peptide 121–134 has no affect on the serum's ability to bind 135–151 and reciprocally, absorption with peptide 135–151 does not affect the binding to 121–134 (Table II). Each peptide contains a strain-specific epitope.

Identification of a Common Determinant in Gonococcal Pili. Even though the immunodominant epitopes of gonococcal pilin are strain specific, a common determinant(s) also has been demonstrated (Fig. 1) to exist within CNBr-2. Peptides chosen to span most of this region of conserved sequence between MS11 and R10 pilin were synthesized as possible epitopes (Table I). Glycine and

TABLE II

*Reaction of Anti-MS11 Pili Sera with Peptides Corresponding to Residues 121–134 and 135–151 Before and After Absorption**

Dilution	Polyclonal sera absorbed with Sepharose			Polyclonal sera absorbed with 121–134-BSA-Sepharose			Polyclonal sera absorbed with 135–151-BSA Sepharose		
	121–134-BSA	135–151-BSA	BSA	121–134-BSA	135–151-BSA	BSA	121–134-BSA	135–151-BSA	BSA
	cpm	*cpm*	*cpm*	*cpm*	*cpm*	*cpm*	*cpm*	*cpm*	*cpm*
1:100	9,483	10,801	428	507	8,769	428	9,408	563	549
1:500	2,229	2,856	145	193	2,062	103	2,352	186	135
1:1,000	1,245	1,450	89	150	1,101	78	1,496	110	75
1:5,000	234	266	47	99	231	45	321	61	64
1:10,000	152	249	37	69	155	26	167	78	88

* Details of the absorption are described in Materials and Methods. Wells were coated with 10 μg/ml of either 121–134-BSA, 135–151-BSA, or BSA, treated with either absorbed or unabsorbed sera, washed, and then exposed to 50,000 cpm of ^{125}I protein A. The values reported are averages of three separate determinations.

cysteine were added to the carboxyl terminus of each peptide to allow them to be conjugated to BSA as previously described. Antisera against both MS11 and R10 pili and against CNBr-2 of each pilin were tested for their ability to bind these conjugates. Anti-whole pili, either MS11 or R10, bound only the region corresponding to residues 48–60 (Fig. 3, *A* and *C*). As expected for a common, immunorecessive determinant, both sera bind equally well, but significantly less than the sera bind the strain-specific epitopes. Neither sera bound peptides corresponding to residues 21–35, 41–50, or 69–84.

A different binding pattern is evident when antisera against the cyanogen bromide–generated fragment, 8–92, of either MS11 or R10 is used (Fig. 3, *B* and *D*). Anti-MS11 CNBr-2 binds the peptide corresponding to residues 69–84 more effectively than 48–60. Anti-R10 CNBr-2 does not bind 48–60 at all. Instead, this serum binds regions corresponding to residues 69–84 and 41–50.

These experiments indicate that an epitope common to both R10 and MS11 pili exists between residues 48–60 and that the immunogenicity of this region is determined by interactions present in intact pili but absent from CNBr-2. Another common determinant, corresponding to residues 69–84, evidently is exposed when CNBr-2 is used an an immunogen.

A solid phase radio-binding assay was performed with the MS11 and R10 anti-whole pili sera to more precisely compare the relative amount of antibodies bound by the common and each of the strain-specific determinants. Table III is a compilation of the results of this experiment. At a 1 to 50 dilution of anti-MS11 pili sera approximately three times as many antibodies are bound by each of the strain-specific epitopes as by the common determinant. Consequently <15% of the antigenicity of whole pili resides in the common determinant, consistent with the data shown in Fig. 1.

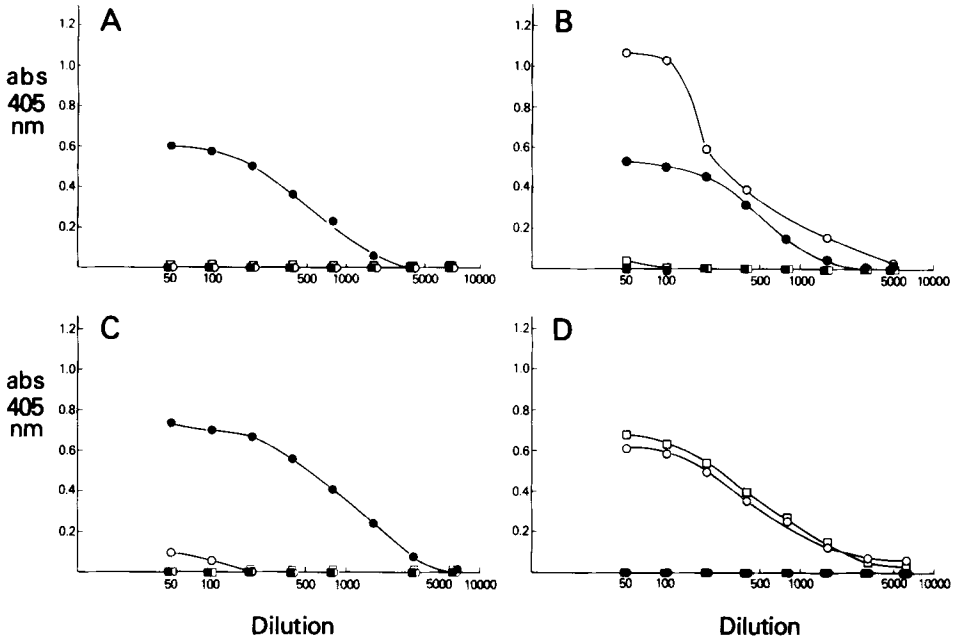

FIGURE 3. Reaction of anti-gonococcal pili sera with peptide-BSA conjugates corresponding to regions of conserved sequence between strains. Anti-MS11 pili (*A*), anti-MS11 CNBr-2 (*B*), anti-R10 pili (*C*), and anti-R10 CNBr-2 (*D*) antisera were assayed for their ability to bind peptides corresponding to residues 21–35 (■), 41–50 (□), 48–60 (●), and 60–84 (○). The enzyme immuno-binding assays were performed as described in Materials and Methods. Each point represents the average of three determinations.

TABLE III

*Relative Binding of Polyclonal Rabbit Sera Against Intact MS11 Pili to Each of the Peptide-BSA Conjugates**

	BSA	21–35	41–50	48–60	69–84	95–107	107–121	121–134	135–151
	cpm	*cpm*	*cpm*	*cpm*	*cpm*	*cpm*	*cpm*	*cpm*	*cpm*
Anti-MS11 1:50	612 ± 12	656 ± 21	810 ± 28	6,519 ± 112	649 ± 15	635 ± 26	1,824 ± 41	17,251 ± 127	18,020 ± 95
Relative %	—	—	—	14.5	—	—	1.5	40.7	42.6

* Wells were coated with 10 μg/ml of the various peptide-BSA conjugates, washed, treated with a 1:50 dilution of anti-MS11 sera, washed, exposed to 50,000 cpm of ^{125}I protein A, washed, and the wells were cut from the plate and counted.

Discussion

Defining the antigenic structure of gonoccocal pili is important for both the design of an efficacious vaccine and for providing molecular details that will lead to a general understanding of how pathogens create antigenic variation to avoid the host immune system.

The experiments described in this paper have partially defined the natural epitopes of gonococcal pili by using synthetic peptides corresponding to linear sequences of the protein. Two strain-specific determinants are located within the disulfide loop near the carboxyl terminus and a single epitope common to both strains R10 and MS11 is present between residues 48 and 60. We recognize that these areas might not constitute the complete antigenic structure of pili because

if there are any determinants composed of nonlinear amino acids, created by the juxtaposition of residues due to either the folding of the pilin molecule or its polymerization, they would not be detected by these reagents. However, an analysis of the regions containing the linear epitopes indicates that they can account for many of the interesting features of the antigenicity of gonococcal pili.

The location of the three peptides containing the antigenic determinants (48–60, 121–134, and 135–151) as well as the five other areas synthesized within the MS11 pilin sequence are shown in Fig. 4. This figure also includes plots of the protein's hydrophilicity and its predicted secondary structure. Table IV compares the sequences of R10 and MS11 pilin in the eight regions synthesized as well as the quantitative predictions for β-turns using the values compiled by Chou and Fasman (15). All known sequence variation between R10 and MS11 pilin occurs in the carboxyl terminal cyanogen bromide–generated fragment (CNBr-3, residues 93–159) (7). The most striking feature of the variations in sequence between R10 and MS11 is that most of the differences occur within or adjacent to predicted β-turns and yet none of the substitutions affect the predicted secondary structure (Table IV).

The initial choice of the peptides synthesized was based on several criteria, including the predicted location of β-turns. The inherent characteristics of reverse turns makes them attractive both as immunogens within synthetic peptides and as possible antigenic determinants in globular proteins. When a synthetic peptide is used as an immunogen, the possible conformations that it can adopt will determine which clones in the B cell repertoire are stimulated to produce antibody. Only if a peptide adopts a secondary structure identical to

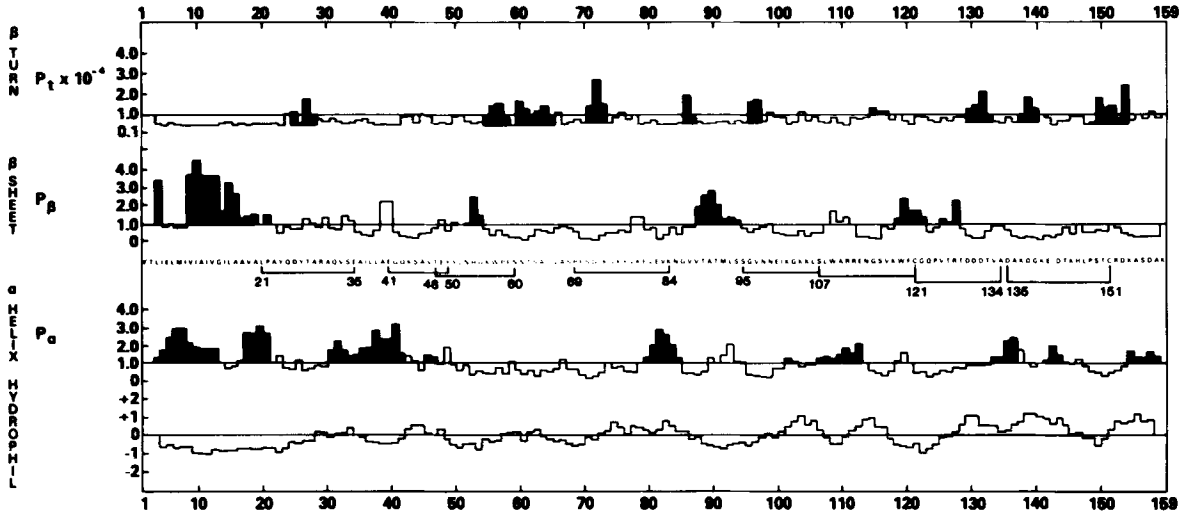

FIGURE 4. Location of the eight oligopeptides within the sequence of MS11 pilin, their predicted secondary structure (15), and their relative hydrophilicity. The relative hydrophilicity of the protein was calculated by a moving average over six residues using the values assigned by Levitt (16). Positive values correspond to the hydrophilic regions. The shaded areas indicate regions of probable conformation.

TABLE IV

Comparison of MS11 and R10 Pilin Sequences in the Regions Synthesized and the Predicted Location of β-Turns

		Sequences	Turn probability*
MS11	21	L P A Y Q D Y T A R A Q V S E 35	1.79×10^{-4}
R10		– – – – – – – – – – – – – – ‡	
MS11	41	E G Q K S A V T E Y 50	0.76×10^{-4}
R10		– – – – – – – – – –	
MS11	48	T E Y Y L N H G K W P E N 60	1.43×10^{-4}, 1.62×10^{-4}§
R10		– – – – – – – – – – – – –	
MS11	69	P P S D I K G K Y V K E V E V K 84	3.10×10^{-4}
R10		[ ]‖	
		+ –	
MS11	95	S G V N N E I K G K K L S 107	0.89×10^{-4}
R10		– N – – K – – – D – – – –	0.84×10^{-4}
MS11	107	S L W A R R E N G S V K W F C 121	0.94×10^{-4}
R10		– – – – K – – A – – – – – – –	0.86×10^{-4}
		+2 +	
MS11	121	C G Q P V T R T D D D T V A 134	2.29×10^{-4}
R10		– – – – – – – A – K – N R T	2.75×10^{-4}
		+ –2 +	
MS11	135	D A K D G K E I D T K H L P S T C 151	1.88×10^{-4}, 1.81×10^{-4}
R10		V – D A [ ]	

* Probability of the underlined four amino acids to be present in a reverse turn based on the algorithm derived by Chou and Fasman (15). Regions whose probability is greater than 0.7×10^{-4} are assigned as turns by this algorithm. Relative charge differences between the two sequences are shown above the indicated position of the MS11 peptide.

‡ – – – indicates sequence identity between MS11 and R10 pilin.

§ Two overlapping turns are predicted in this region, HGKW and WPEN.

‖ [ ] indicates unsequenced regions of R10 pilin.

that it assumes in the intact protein will it both bind sera generated against the intact protein and elicit antibodies that cross-react with that protein. Even if the antibody-combining site induces the peptide to adopt the correct conformation, a peptide having a higher probability to preexist in that conformation will have the lowest energy requirements for binding. Of the three common secondary structures present in proteins, β-pleated sheets, α-helices, and reverse or β-turns, we believe reverse turns have the highest probability of being present in an isolated oligopeptide <15 residues in length. Individual strands of a sheet require interactions with the adjacent strands to adopt that conformation and to be subsequently stabilized (17). Helices can form in an isolated peptide, but the peptide must be long enough to generate sufficient intrahelical interactions to stabilize the conformation (18). Further stabilization often results from a particular face of a helix interacting with adjacent regions of the protein (17). In

contrast, the forces involved in the formation of a reverse turn are thought to be contained within the primary structure of the peptide (19). Therefore, a peptide containing a region with high turn probability will be more likely to be identical to the corresponding region in the intact protein than regions predicted to be sheets or helices.

Turns also are present in immunologically interesting regions in proteins. A number of investigators have postulated that turns exist as a means of keeping a protein globular by reversing the direction of a sheet or helix back into the center of a protein, which results in the turn being present on the surface of a protein (20–24). Turns also are likely regions for advantageous mutations to occur within microbial proteins exposed to host immunity. Unlike sheets and helices, the amino acid side chains in turns interact with the solvent and not with other regions of the protein. Consequently, as long as the substitution of an amino acid maintains the turn there is no need to mutate a second amino acid to conserve the correct tertiary structure of the protein, as might be the case if the mutation occurs in a helix or sheet.

The experiments reported in this paper support this proposal. Both of the peptides containing strain-specific determinants (121–134 and 135l–151) have a predicted turn around which the sequence variations are localized (Table IV). Each peptide also contains a turn within which there exists a position where one strain has a lysine, whereas the other contains an aspartic acid. Such a variation in sequence involving a double charge change could explain the total absence of cross-reactivity of the complementary antibody-combining sites. Another feature of the strain-specific determinants is that they are localized within a disulfide loop, a region of the protein previously shown not to be involved in either pilin polymerization or receptor binding (8). Single disulfide loops are also found in *Moraxella bovis* (G. K. Schoolnik, unpublished observations), *Pseudomonas aeruginosa* (25), and common and Gal-Gal binding *Escherichia coli* (26, 27) pili. We believe that the location of the principal epitopes within the disulfide loop is part of a sophisticated molecular design that enables the organism to segregate its principal antigenic determinants in a region distinct from the residues involved in receptor binding and subunit polymerization. By using a disulfide loop, we propose that the molecule has evolved a domain that (*a*) prominently orients the region on the surface of the molecule, perhaps even projecting out from the polymeric structure, resulting in its increased antigenicity and immunogenicity; (*b*) allows amino acid substitutions, additions, and deletions within the antigenic determinants, thereby maximizing antigenic variation, without disrupting areas critical for the biological role of the protein; and (*c*) permits an antibody to bind pili and yet not interfere with gonococcal adhesion to the mucosal cell surface. The highly variable antigenic determinants of influenza virus neuraminidase recently have been shown to be designed in a similar fashion. They are located in β-turns, in a region distinct from the active site, which enables antibodies to bind to the epitopes without inhibiting the enzyme (28).

In addition to making the regions within the disulfide loop immunodominant, the design of pilin also has resulted in the observed regions involved in receptor binding being immunorecessive. Our experiments have shown that the small, cross-reactive antibody fraction of anti-pili sera is directed at an epitope located

between residues 48 and 60. The poor immunogenicity of this region compared with the strain-specific determinants explains why pili from a single gonococcal strain are not suitable immunogens for use as a vaccine. In contrast, the central cyanogen bromide–generated fragment (CNBr-2, residues 8–92) elicits antiserum that cross-reacts well with heterologous gonococcal pili (8, Fig. 1). Experiments in this paper indicate that the principal epitope of this fragment is not between residues 48 and 60, but rather between residues 69 and 84, an area not recognized by sera against intact pili. The immunogenicity of the region corresponding to residues 69–84 apparently is greatly reduced in the intact protein.

The immunogenicity and functional properties of antibodies engendered by the synthetic peptides described in this report and to the fragments from the conserved, receptor-binding region of the pilin sequence currently are being studied to identify suitable components for a vaccine.

Summary

The antigenic structure of gonococcal pilin, strain MS11 (Tr), was investigated by assaying the binding of antisera engendered by intact pili from strains MS11 and R10 and their two major cyanogen bromide–generated fragments, CNBr-2 (residues 9–92) and CNBr-2 (residues 93–159), to synthetic peptides corresponding to the amino acid sequence of MS11 pilin. Four peptides were synthesized corresponding to regions of sequence variation between MS11 and R10 gonococcal pilin. Antisera against the homologous pilus filament and against its CNBr-3 fragment bind peptides equivalent to residues 121–134 and 135–151, which comprise the 30 amino acid disulfide loop near the carboxyl terminus of the protein. Heterologous pili antisera did not bind these peptides. Absorption studies proved that each peptide contained an independent, strain-specific epitope. Synthetic peptides corresponding to regions of identical sequence between MS11 and R10 pilin were used in similar binding experiments to localize a weakly immunogenic, common determinant between residues 48 and 60. <15% of the antibodies raised against intact pili were directed at this site. Antisera raised against MS11 or R10 CNBr-2 bind a separate peptide, residues 69–80. This region is immunogenic only as a fragment, not in the intact pilus filament.

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