ANTIGENIC ANALYSIS OF GONOCOCCAL PILI USING MONOCLONAL ANTIBODIES

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The attachment of gonococci to mucosal surfaces is of primary importance in their interactions with the genito-urinary tract of the human host. This process may be, in part, mediated by pili, which are proteinaceous appendages emanating from the bacterial cell surface. Observations by Kellogg et al. (1) suggested that certain colonial morphological phenotypes are associated with virulence. Subsequently, Swanson et al. (2) demonstrated that gonococci giving rise to the colonial phenotype associated with virulence carry pili on their surface. Subsequently, piliated gonococci have been shown to agglutinate erythrocytes (3), and attach to spermatozoa (4), epithelial cells (5), and human fallopian tubes in organ culture (6, 7).

Pili are filaments, 1–4 μm in length, composed of repeating, identical subunits of the protein pilin. Pilin demonstrates a variable apparent molecular weight, depending on type, of 17,500–21,000 by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (8). Gonococcal pilins possess a single intramolecular disulfide bond and two methionine residues, which allows for cleavage by cyanogen bromide into three peptide fragments (CB-1, 2, and 3) (9). The CB-2 fragment (residues 8–92 in strain MS11 pilin) possesses the erythrocyte-binding domain, as evidenced by its ability to compete with intact pili in a hemagglutination assay. Peptide mapping of CB-2 fragments from serologically distinct pilin (strains MS11 and R10) demonstrates homology in this area of the protein (9). The complete amino acid sequence of MS11 pilin has been obtained, as well as the first 59 amino acids of R10 pilin (10). Comparison of these sequences demonstrates identity through amino acid 59, providing additional evidence that the amino-terminal half of gonococcal pilin is highly conserved. Conversely, peptide mapping of CB-3 fragments (residues 93–159 in MS11 pilin) demonstrates significant differences between pilin types, indicating that the carboxy-terminal portion of the protein contains the variable domain (9).

The distinct serological heterogeneity of gonococcal pili has been demonstrated by a number of investigators (8, 11). Immunization of rabbits and mice with purified pili elicits immune responses that are strikingly type specific (8). Vaccination of humans with purified pili produced primarily type-specific anti-

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bodies, thus limiting the usefulness of this approach (12). Since the peptide mapping and sequence data of pilin indicate structural homology in the amino-terminal half of the protein, it is remarkable that these common determinants escape detection by the immune system. Because of the interest in developing effective vaccines for gonorrhoea, we have produced and characterized a bank of mouse monoclonal antibodies for the elucidation of epitopes in the pilin protein.

Materials and Methods

Bacterial Strains and Media. *Neisseria gonorrhoeae* strains MS11 and R10 have been maintained in the laboratory for several years, and were isolated from patients with uncomplicated, anterior urethritis. Isogenic piliated and nonpiliated clones of each strain were maintained by daily single-colony passage on solid typing medium as described previously (13).

Isolation of Pili. Pili were isolated by a modification of the method of Brinton et al. (8). Briefly, piliated gonococci were inoculated onto 150 × 15 mm solid agar plates (14) and grown in candle jars for 19 h. All subsequent steps were done at 4°C. Each agar plate was flooded with 0.15 M ethanolamine-HCl buffer, pH 9.8 and the colonies were scraped into a Sorvall Omnimixer cup (400 ml) (Sorvall Instruments Div., Du Pont Co., Newton, CT) The gonococcal suspension was blended at 2,000 rpm for 10 min. Bacteria were removed from the suspension by centrifugation at 12,000 *g* for 30 min and were reextracted with ethanolamine buffer under identical conditions. The supernates were pooled and centrifuged at 35,000 *g* for 60 min. Ammonium sulfate (20%) was slowly added to the supernate to a final concentration of 10%. The solution was mixed for 12 h and then centrifuged at 12,000 *g* for 60 min. The pellet containing aggregated pili was redissolved in ethanolamine buffer and separated from the insoluble debris by centrifugation at 17,000 *g* for 60 min. The solubilized pili were dialyzed exhaustively against 0.05 M Tris-HCl, pH 8.0, containing 0.15 M NaCl (Tris-saline). The aggregated pili were sedimented by centrifugation at 17,000 *g* for 60 min, and redissolved in ethanolamine buffer. The solubilization-aggregation procedure was repeated twice. The purified pili were stored in ethanolamine buffer at 4°C.

Preparation of Pilin Fragments. Cyanogen bromide cleavage of R10(Tr) pilin at methionine residues was performed as described by Schoolnik et al. (10). CB-2 and CB-3 (respectively, residues 8–92 and 93–159 of the MS11(Tr) pilin sequence) were purified by reverse phase, high pressure liquid chromatography (10). Peptide TC-2 (corresponding to residues 31–111 of the MS11(Tr) pilin sequence) was generated by tryptic hydrolysis of citraconylated R10(Tr) pilin and the deblocked peptide was purified by high pressure, steric exclusion liquid chromatography (10).

Synthesis of Pilin Peptide Analogues. The peptides shown in Table I were synthesized by solid phase techniques (15) on a Beckman 990B peptide synthesizer (Beckman Instruments, Inc., Fullerton, CA) using commercially available *t*-Boc-protected amino acids and resins obtained from Peninsula Laboratories (Belmont, CA). The family of coterminal peptides (see Fig. 1) was synthesized simultaneously by removing aliquots of resin during the synthesis, and deprotecting them separately. For immunological assays, each peptide was conjugated to bovine serum albumin through the reduced sulfhydril of the carboxy-terminal cysteine, using heterobifunctional coupling agents (16).

Immunological Methods. Enzyme-linked immunosorbent assays (ELISA)[1] (17) were performed in Immulon II microtiter plates (Dynatech Laboratories, Inc., Alexandria, VA) sensitized overnight with pili CB-3 or CB-2, at a concentration of 1 μg/ml, TC-2 at 0.2 μg/ml, and synthetic peptide–bovine serum albumin conjugates at 20 μg/ml, all in 0.1 M Tris-HCl, pH 9.8. After sensitization, the plates were washed six times with 0.9% NaCl and 0.05% Brij-35 (Pierce Chemical Co., Rockford, IL) (NaCl-Brij). Supernates containing

[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline.

TABLE I

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

Sequence	Location
LPAYQDYTARAQVSEGC*	21 35
EGQKSAVTEYGC	41 50
TEYYLNHGKWPENGC	48 60
PPSDIKGKYVKEVEVKGC	69 84

* The underlined residues do not exist in MS11 pilin. The additional glycine and cysteine residues were added to serve as spacer and to facilitate covalent attachment to carrier molecules, respectively.

monoclonal antibodies were added in 0.01 M phosphate, pH 7.2, 0.15 M NaCl, and 0.05% Brij-35 (PBS-Brij) and incubated 4 h at room temperature. The microtiter plates were again washed six times in NaCl-Brij, then incubated overnight with alkaline phosphatase (Sigma Chemical Co., St. Louis, MO)-conjugated goat anti-mouse IgG (Tago, Inc., Burlingame, CA) in PBS-Brij (18). After six washings in NaCl-Brij, *p*-nitrophenyl phosphate (Sigma 104 phosphatase substrate; Sigma Chemical Co.) was added at a concentration of 1.0 mg/ml in 1 M Tris-HCl, pH 9.8, 3 mM $MgCl_2$, and incubated at 37°C for 1 h. The titers were defined as the reciprocal of the antibody dilution producing half-maximal absorbance at 405 nm. Assays were done in duplicate and the geometric means were reported.

Competitive inhibition ELISA was performed in Immulon II microtiter plates sensitized overnight with intact pili at a concentration of 1 μg/ml in 0.1 M Tris-HCl, pH 9.8. The TC-2 fragment of R10 pilin was added to dilutions of PEG-concentrated monoclonal supernatants in PBS-Brij and allowed to react overnight at 4°C. The monoclonal antibodies were diluted to concentrations that gave 30–70% of the maximal absorbance in a direct ELISA. The mixtures containing inhibitor and monoclonal antibody were added to the washed, sensitized microtiter plates and allowed to react 4 h. Plates were then processed as described for the ELISA.

Preparation of Monoclonal Antibodies. BALB/c mice were injected with 100 μg of purified gonococcal pili (strain R10 or MS11) in incomplete Freund's adjuvant intraperitoneally. 4 wk later, they were given an additional 100 μg of intact pili in saline intraperitoneally. On day 56, the mice were examined for anti-pili antibody production by ELISA screening, and mice with titers >900-fold compared with preinjection sera were given 100 μg i.p. of pili in distilled water. 4 d later, cells were obtained from the spleens of three mice, fused with BALB/c NSI/1 cells, and distributed to fourteen 96-well tissue culture plates in hypoxanthine, aminopterin, thymidine (HAT) medium (19). 14 d later, the supernates of each well were tested for reactivity with pili by direct ELISA. The cells of reactive wells were subcloned by limiting dilution and propagated in Dulbecco's modified Eagle's medium plus 20% fetal calf serum. The clones were frozen at −70°C and the supernatants containing monoclonal antibody were precipitated with PEG (final concentration of 15%) and resuspended in 200 mM sodium phosphate, pH 8.0 with 0.02% sodium azide.

Results

Reactivity of Monoclonal Antibodies with Intact Pili. Two sets of mice were immunized, with pili from strain MS11 or R10, respectively. When the animals showed good serum-immune responses they were sacrificed and their spleen cells used in two separate fusion experiments. Hybridoma supernatants were screened

for anti-pilin activity by ELISA using the homologous, purified pili as antigen. The initial screening found 66 and 44 positive wells for R10 and MS11 pili, respectively. After subcloning of positive wells, 33 clones were isolated that produced monoclonal antibodies to R10 pilin. After additional cloning, 18 of the R10 pili–reactive clones survived. Due to a technical problem, all but one of the MS11-reactive clones were lost, the surviving clone being 1E8/G8. The titers of PEG-concentrated supernatants from the 19 clones to R10 and MS11 pili are shown in Table II. As expected, the majority of the clones produced type-specific antibodies that did not cross-react with heterologous pili. The 16 R10-reactive clones listed in the first two groups in Table II demonstrated significant anti–R10 pili reactivity and no activity towards MS11 pili. The third group of clones, however, did demonstrate reactivity to the heterologous pili type. Certainly, 13D6/B10 was only slightly reactive with MS11 pili and was therefore a marginal member of this group. The clones 9B9/H5 and 1E8/G8 were equally reactive with either pili type and represented the clones of most interest.

Reactivity of Monoclonal Antibodies with Linear Determinations. To determine if the epitopes of these monoclonal antibodies could be localized to specific areas of the pilin protein, various fragments of R10 pilin were used as antigen. The

TABLE II
Immunological Reactivity of Monoclonal Antibodies

Antibodies	Antigens		
	R10 Pili	MS11 Pili	R10 CB-3
Group 1			
4D6/F8	14,700*	<10	6,900
5B12/F11	6,400	<10	9,700
5D8/G11	3,700	<10	3,400
5E9/7C	3,700	<10	5,600
10A4/E9	19,400	<10	18,100
10C7/G9	9,700	<10	7,900
10D9/C11	18,100	<10	8,200
10F2/A5	7,350	<10	7,900
12F1/F5	10,400	<10	19,400
13G7/B4	9,700	<10	12,800
Group 2			
9H5/D5	12,800	<10	630
9G8/D4	3,200	<10	300
4B8/F11	19,400	<10	300
2D7/G10	10,800	<10	140
2B6/E6	5,600	<10	140
7A10/G5	11,100	<10	<2
Group 3			
9B9/H5	300	700	<2
13D6/B10	4,850	90	18
1E8/G8‡	9,400	7,300	<2

* The numbers indicate reciprocal ELISA titers.
‡ Clone 1E8/G8 is derived from the spleen of a mouse immunized with MS11 pili.

CB-3 fragment (residues 93–159 in MS11 pilin) of gonococcal pilin has been characterized as the carboxy-terminal portion containing the source of structural heterogeneity. The R10 clones (group 1 of Table II) were quite reactive with this fragment. In these cases, the titers to R10 CB-3 and R10 pili were similar, suggesting their epitopes to be linear determinants, or perhaps nonlinear determinants not destroyed by cyanogen bromide cleavage. The second group of R10-reactive clones, however, were only minimally reactive with R10 CB-3. This suggests that their epitopes reside in the CB-3 fragment but were partially destroyed upon cleavage. The cross-reactive antibodies of group 3 did not demonstrate anti-R10 CB-3 activity.

To probe the epitopes of the cross-reactive antibodies, a similar analysis was attempted using the R10 CB-2 fragment. CB-2 from MS11 pilin consists of residues 8–92 and is known to be homologous with the R10 pilin sequence at least through residue 59 (20). We therefore assumed the R10 CB-2 fragment to be similar and to contain most of the cross-reactive epitopes. Unfortunately, CB-2 fragments displayed anomalous behavior in ELISA. This was demonstrated by using a rabbit antiserum produced by immunization with purified R10 CB-2 in ELISA. The titer of this serum against R10 pili was 500,000, compared with only 50,000 against R10 CB-2. CB-2 fragments have been shown to form high molecular weight aggregates in aqueous solution, probably due to the hydrophobic nature of residues 8–30 (21). For this reason, a pilin fragment of greater solubility was desired.

A solution to this problem was achieved using a similar fragment produced by tryptic cleavage of citraconylated pilin that cleaves proteins on the carboxyl side of arginine residues (22). The TC-2 fragment of MS11 pilin consists of residues 31–111 and, on the basis of prior incomplete sequence analysis of R10 pili, we made the assumption that the sequence of R10 TC-2 was similar (20). The R10 TC-2 fragment demonstrated a reliable antigenicity in ELISA and the rabbit anti-R10 CB-2 serum titered at 632,000. When the bank of monoclonal antibodies was tested for anti-R10 TC-2 reactivity, only 2 of 19 were positive. These were 9B9/H5 and 1E8/G8, both members of the cross-reactive group 3. In each case their reactivities were weak: the maximum absorbances were 0.750 and 0.230 for 9B9/H5 and 1E8/G8, respectively. We considered that the solid phase nature of the direct ELISA could interfere with antibody binding, so the R10 TC-2 fragment was used as a competitive inhibitor of anti-R10 pili activity. Microtiter plates were sensitized with R10 pili and reacted with various dilutions of supernatants from the clones in group 3 that had been pretreated with 400 ng of R10 TC-2. Compared with the untreated controls, only the 9B9/H5 antibodies displayed a loss of reactivity towards R10 pili. In two separate experiments, the anti-R10 pili activity of a 1:1000 dilution of 9B9/H5 supernatant was inhibited 66 and 62% by the R10 TC-2. Significant inhibition of 1E8/G8 or 13D6/B10 was not observed. These data would localize the epitope of 9B9/H5 in the TC-2 region of the pilin protein.

Mapping of Epitopes with Synthetic Peptides. To more specifically localize epitopes of the cross-reactive antibodies, synthetic peptides mimicking areas of the CB-2 fragment of MS11 pilin were produced. Because the complete amino acid sequence of MS11 pilin is known, four peptides corresponding to residues 21–

35, 41–50, 48–60, and 69–84 were synthesized (Table I) and used in ELISA. The ability of these peptide-BSA conjugates to bind to microtiter plates was confirmed with a rabbit anti-MS11 CB-2 serum. Microtiter wells were sensitized with the peptides and reacted with the rabbit serum. Peptides 41–50 and 69–84 were reactive at titers >20,000. Peptides 21-35 and 48-60 were less reactive, indicating either a marginal association with the polystyrene or a weak immunogenicity of these areas of the pilin protein. When these peptides were used to screen the bank of monoclonal antibodies, all proved negative except 9B9/H5, whose titer with peptide 69–84 was 7,350. This was an interesting result because 9B9/H5 was 10-fold less reactive with intact R10 pili, suggesting that the epitope of 9B9/H5 is a linear determinant and is probably partially masked in the intact pilin protein.

To more finely map the epitope of the 9B9/H5 antibody, three additional peptides reflecting portions of peptide 69-84 were synthesized. Peptides 79-84, 76-84, 72-84, and 69-84 were produced and conjugated to bovine serum albumin. The reactivity of 9B9/H5 supernatant in ELISA with these peptide-BSA conjugates is shown in Fig. 1. The synthetic, 15 amino acid peptide 69-84 elicited a titer of 1820, whereas the 12 amino acid peptide 72-84 titered at ~1300. This decrease in activity suggests that the tripeptide Pro-Pro-Ser (residues 69–71) contributes to the epitope of 9B9/H5 antibody. Reactivity of the monoclonal antibody with peptide 76-84, an eight amino acid peptide, was greatly reduced

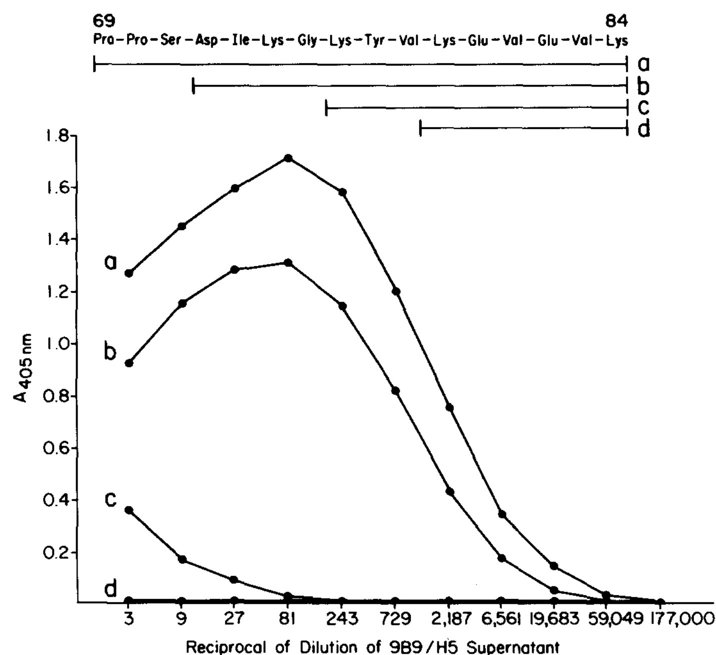

FIGURE 1. ELISA reactivity of monoclonal antibody 9B9/H5 with synthetic peptides conjugated to BSA. The amino acid sequence of each peptide appears at the top. The numbers represent the position in the MS11 pilin sequence. The peptide-BSA conjugates were used to sensitize microtiter plates, and dilutions of 9B9/H5 hybridoma supernatant were used as primary antibody. Alkaline phosphatase–conjugated goat anti-mouse IgG diluted 1:1000 was used as the secondary antibody. Each point represents the mean of duplicate determinations.

to a titer of 9, indicating that the sequence Asp-Ile-Lys-Gly (residues 72–75) constitute the immunodominant area of the epitope. Since peptide 76-84 demonstrated slight reactivity, residue 76 (Lys) may represent the carboxy-terminal end of the epitope. The six amino acid peptide 79-84 was not reactive with 9B9/H5 antibody.

Discussion

To summarize the ELISA results, the 19 pilin-reactive clones can be arranged into three groups. The first group demonstrated excellent reactivity with R10 pili and CB-3 and were not reactive with MS11 pili. These antibodies were, therefore, directed towards type-specific epitopes in the 50 carboxy-terminal amino acids of R10 pilin. The second group demonstrated similar anti-R10 pili reactivities as the first group, yet were only slightly reactive or not reactive with the CB-3 fragment of R10 pili. Their reactivity with CB-3 was at least 10-fold less than that of group 1. This was most likely due to conformational changes upon cyanogen bromide cleavage. These antibodies could be classified as being directed towards type-specific, nonlinear epitopes. The third group consisted of the remaining three clones that demonstrated cross-reactivity between pilin types. Their lack of reactivity with the CB-3 fragment indicated that their epitopes probably lie in the amino-terminal half of the protein. Confirmation of this was achieved with 9B9/H5, whose reactivity was inhibited with the TC-2 fragment of R10 pili.

The propensity of animals, including humans, immunized with gonococcal pili to produce type-specific, anti-pili antibodies has been well documented (8, 16). A study by Virji and Heckels (23) reported the production of 10 monoclonal antibodies reactive with pili from gonococcal strain P9 (23). Only two of these antibodies demonstrated reactivity with heterologous pilin types. It was, therefore, not surprising that 16 of the 19 monoclonal antibodies described in this study were type specific. The mechanisms whereby the pilin protein displays this type-specific immunodominance are unknown, although the advantages to the gonococcus are obvious. Since 10 of the type-specific antibodies reacted with the R10 CB-3 fragment (group 1), it seems that synthetic peptides reflecting parts of this fragment could be used to locate these immunodominant epitopes. This will become possible once the amino acid sequence of R10 pilin is completed. The second group of monoclonals indicated that epitopes defined by conformational restraints are not uncommon in the pilin protein. This group of monoclonal antibodies may prove useful in the elucidation of tertiary structure in the carboxy-terminal portion of the protein.

A major goal of this study was the description of species-specific, cross-reactive epitopes for vaccine research. It seems possible that a synthetic peptide encompassing a species-specific epitope could serve as an effective vaccine for gonorrhoea. For this reason, the cross-reactive monoclonals of group 3, especially 9B9/H5 and 1E8/G8, were of great interest.

The epitope of the immunoglobulin produced by the 9B9/H5 clone was shown to be a linear determinant, for the antibody was more reactive with a 15 amino acid peptide than intact pilin or pilin fragments containing this segment. This was most likely due to a partial masking of the epitope by the tertiary protein

structure. The ELISA data with the TC-2 fragment of R10 pilin supports this idea, since TC-2 was a more effective antigen for the 9B9/H5 antibody in solution (competitive inhibition ELISA) than when bound to polystyrene (direct ELISA). The flexibility of the TC-2 fragment in solution might be greater, leading to an increased exposure of buried epitopes.

The use of synthetic peptides homologous to the amino acid sequence of MS11 pilin enabled the fine mapping of the 9B9/H5 epitope. Initially, this epitope was localized to the area of the protein near amino acids 69–84. This synthetic peptide was an effective antigen alone or as a BSA conjugate in direct ELISA. The reactivity of 9B9/H5 antibody with shorter peptides indicated that the epitope must lie between amino acids 69 and 76, with residues 72–75 composing the primary structural entity of the epitope. It is possible that the epitope extends beyond the amino-terminal side of residue 69, but this seems unlikely due to size restrictions for a linear epitope, ~6 residues (24). The fine mapping of this epitope using peptides that mimic known sequences demonstrated the utility of this approach.

The 1E8/G8 antibody differs from 9B9/H5 in two significant properties. First, 1E8/G8 demonstrates excellent reactivity with intact gonococcal pili. Second, the epitope responsible seems nonlinear, since none of the peptide fragments or synthetic peptides were strongly antigenic for the 1E8/G8 antibody. The weak reactivity of this monoclonal antibody with the R10 TC-2 fragment suggests that the epitope is located in this area of the protein. Conformational changes of the TC-2 fragment could explain the loss of the epitope. The 1E8/G8 antibody has proven to be most useful as an immunological reagent. Because of its strong reactivity with gonococcal pili, it has been used as a probe for pili in Western blotting of electrophoretically separated pilin and as the primary antibody in indirect immunofluorescence of intact organisms. The fact that 1E8/G8 reacts with monomeric pilin in Western blots indicates that its epitope is not destroyed by SDS and that it is not intermolecular. The fact that this antibody cross-reacts so well with intact pili or pilin makes elucidation of its epitope desirable for vaccine research.

Summary

A bank of mouse monoclonal antibodies has been produced with reactivity to gonococcal pili to investigate epitopes of the pilus structural protein, pilin. Pili of *Neisseria gonorrhoeae* strains R10 and MS11 were used as immunogens to elicit 19 monoclonal antibodies reactive with the homologous pili type in ELISA. Of the 19 antibodies, 16 demonstrated type-specific reactivity and 3 were cross-reactive with heterologous pili. Reactivity of the antibodies with the carboxy-terminal, cyanogen bromide fragment (CB-3) of R10 pilin allowed their classification into three groups. The first group (10 antibodies) were R10 specific and equally reactive with the R10 CB-3 fragment. The second group (6) were also type specific but demonstrated poor reactivity with the CB-3 fragment. This suggested that the epitopes of the first group are linear, and those of the second group, nonlinear. The third group (3), consisting of the cross-reactive antibodies, were not reactive with the CB-3 fragment. Two of the antibodies in group 3 were examined in detail to localize their epitopes. The epitope of one, 9B9/H5,

was shown to be a linear determinant. This antibody was reactive with a fragment of MS11 pilin (residues 31–111) and to a synthetic peptide representing residues 69–84 in MS11 pilin. The epitope was more finely mapped, with shorter synthetic peptides conjugated to bovine serum albumin, to an eight amino acid segment (residues 69–76). The epitope of 1E8/G8, a strongly reactive antibody, proved elusive to this type of analysis and probably results from conformational restraints. The significance of species-specific epitopes in the pilin protein is discussed.

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