INFECTION AND IMMUNITY, Feb. 1996, p. 644–652 Vol. 64, No. 2
0019-9567/96/$04.00+0

Assembly and Antigenicity of the *Neisseria gonorrhoeae* Pilus Mapped with Antibodies

KATRINA T. FOREST,[1] SUSAN L. BERNSTEIN,[1] ELIZABETH D. GETZOFF,[1] MAGDALENE SO,[1]† GORDON TRIBBICK,[2] H. MARIO GEYSEN,[2]‡ CAROLYN D. DEAL,[1]§ AND JOHN A. TAINER[1]*

Department of Molecular Biology, The Scripps Research Institute, La Jolla, California 92037,[1] and Chiron Mimotopes Pty. Ltd., Clayton, Victoria 3168, Australia[2]

Received 24 October 1994/Returned for modification 16 January 1995/Accepted 28 November 1995

The relationship between the sequence of *Neisseria gonorrhoeae* pilin and its quaternary assembly into pilus fibers was studied with a set of site-directed antibody probes and by mapping the specificities of antipilus antisera with peptides. Buried and exposed peptides in assembled pili were identified by competitive immunoassays and immunoelectron microscopy with polyclonal antibodies raised against 11 peptides spanning the pilin sequence. Pili did not compete significantly with pilin subunits for binding to antibodies against residues 13 to 31 (13–31) and 18–36. Pilus fibers competed well with pilin protein subunits for binding to antibodies raised against peptides 37–56, 58–78, 110–120, 115–127, 122–139, and 140–159 and competed weakly for antibodies against residues 79–93 and 94–108. Antibodies to sequence-conserved residues 37–56 and to semiconserved residues 94–108 preferentially bound pilus ends as shown by immunoelectron microscopy. The exposure of pilus regions to the immune system was tested by peptide mapping of antiserum specificities against sets of overlapping peptides representing all possible hexameric or octameric peptides from the *N. gonorrhoeae* MS11 pilin sequence. The immunogenicity of exposed peptides incorporating semiconserved residues 49–56 and 121–126 was revealed by strong, consistent antigenic reactivity to these regions measured in antipilus sera from rabbits, mice, and humans and in sera from human volunteers with gonorrhea. The conservation and variation of antigenic responses among these three species clarify the relevance of immunological studies of other species to the human immune response against pathogens. Overall, our results explain the extreme conservation of the entire N-terminal one-third of the pilin protein by its dominant role in pilus assembly: hydrophobic residues 1–36 are implicated in buried lateral contacts, and polar residues 37–56 are implicated in longitudinal contacts within the pilus fiber.

Neisseria gonorrhoeae is a gram-negative bacterium that causes the sexually transmitted disease gonorrhea. The first step in the infection, the attachment of the gonococcus to host epithelial cells, is mediated at least in part by pili (5), long (0.5 to 4 μm) hair-like (diameter, 60 Å [6.0 nm]) protein filaments on the bacterial cell surface (31). Each pilus is formed by the specific association of thousands of pilin protein subunits and apparently minor amounts of pilus-associated proteins. The pilin protein (~18kDa) varies in sequence with serotype and consists of approximately 160 amino acids (27). Antigenic variation occurs predominantly in the C-terminal region as multiple residue insertions and deletions as well as single amino acid changes (11). The highly conserved N-terminal sequence of gonococcal pilin is homologous to other type IV bacterial pilin sequences, including those from *Neisseria meningitidis* (12), *Pseudomonas aeruginosa* (26), *Moraxella nonliquefaciens* (7), and *Dichelobacter nodosus* (17). All of these proteins have the unusual posttranslational modification *N*-methyl-phenylalanine at the amino terminus. Electron micrographs of negatively stained pili from *N. gonorrhoeae* (31), *D. nodosus* (40), and *P. aeruginosa* (2, 43) indicate that these bacterial pili also share gross overall structural features. Thus, results on exposed and buried regions of *N. gonorrhoeae* pili may be applicable to the entire class of type IV pili.

Because the pilus is a major virulence factor of the gonococcus (21), many attempts to produce an antigonococcal vaccine have concentrated on this surface antigen (3, 28, 37, 38). The success of these efforts has been thwarted by the extensive antigenic variation of pilin coupled with the apparent immunorecessive nature of its sequence-conserved regions. Presentation to the immune system of a set of peptides representing sequence-conserved regions might elicit neutralizing antibodies effective against all gonococci, provided that these same peptides are accessible on the intact gonococcus. As many experiments are done with rabbit polyclonal or mouse monoclonal antibodies, it is worthwhile to compare rabbit and mouse responses with the human response to pili and to actual infection. Although the value of mapping human antiserum specificities against peptides representing a single pilin sequence is limited by pilin's tendency to undergo antigenic variation upon infection, it is important because only humans are infected by *N. gonorrhoeae*. Thus, despite limitations on interpretation due to antigenic variation, regions recognized by human antisera likely represent exposed and immunogenic regions in vivo which are relevant to understanding pilus assembly and potential vaccine design.

From the standpoint of molecular biology, the gonococcal pilus presents intriguing questions regarding the structural basis for molecular assembly and recognition. The gonococcal pilus, constructed primarily of a single protein, performs several functions: assembly into fibers, epithelial cell binding, and perhaps nucleic acid binding during transformation (6). Sequence changes in the pilin protein that generate the antigenic

* Corresponding author. Phone: (619) 554-8119. Fax: (619) 554-6880.

† Present address: Oregon Health Sciences University, Department of Molecular Microbiology and Immunology, Portland, OR 97201-3098.

‡ Present address: Glaxo Research Institute, Research Triangle Park, NC 27709.

§ Present address: NIAID, STD Branch, Bethesda, MD 20892-7630.

diversity of pili must fit the constraints imposed by both structure and function; gonococci with nonassembled or dysfunctional pili would likely be avirulent and, thus, selected against in vivo. The results presented here on the assembly and antigenicity of the *N. gonorrhoeae* pilus should reveal the locations of accessible regions both for antipeptide antibodies and for the immune response to the pilus. This information provides constraints that should aid construction of an atomic resolution pilus model once the crystal structure of a pilin subunit becomes available.

MATERIALS AND METHODS

Pilin purification. Pilin was purified by following a modified version of Brinton's method (3, 19) from MS11 variant C30 (29). To minimize antigenic variation, gonococci were stored initially as many individual frozen stocks in 20% glycerol with Trypticase Soy Broth (BBL Microbiology Systems). Cells were revived from a previously unthawed stock vial directly on solid media (GC media and Agar Noble; Difco) supplemented with 22.2 mM glucose, 0.7 mM L-glutamine, 0.6 μM thiamine, and 12.4 μM ferric nitrate, incubated at 37°C under 5% CO_2 in 100-mm-diameter disposable petri plates. The MS11 stocks were not passaged between pilin preparations. Piliated colonies were passaged after 18 to 22 h of growth (chosen to maximize pilin protein yield). After 18 h of growth, gonococci from batches of 50 plates were harvested into 20 ml of cold freshly prepared buffer (50 mM 2-[*N*-cyclohexylamino]-ethanesulfonic acid [CHES] buffer, pH 9.5, 1 mM dithiothreitol) on ice with a glass spreader bar and turntable. Pili were sheared from cells by vortexing the cell-buffer suspension in three 1-min bursts separated by 30-s intervals during which the suspension was cooled on ice.

The following purification steps were all done at 4°C. Whole cells and outer membrane aggregates were removed by centrifugation at 8,000 × *g* for 20 min. To reaggregate pilus fibers from single fibers in the supernatant, the pH was lowered by overnight dialysis against Tris-buffered saline (TBS) (50 mM Tris(hydroxymethyl)aminomethane, 150 mM NaCl [pH 7.5], 0.02% NaN_3). Aggregated pilus fibers were pelleted by centrifugation at 8,000 × *g* for 20 min, and the pellet was resuspended by disassociation in 50 mM CHES buffer at pH 9.5 and centrifuged to remove contaminants; the dialysis and centrifugation steps were repeated for the supernatant from 3 to 10 times. The final pellet consisting of purified pilus fibers was resuspended in buffer appropriate for subsequent experiments. Pili in Tris-buffered saline at pH 7.5 are fibers and bundles of fibers as seen by electron microscopy and as evidenced by the fact that the pili are removed from the solution by centrifugation. Pili in low salt buffer at pH 9.5 form single fibers which remain in solution as evidenced by electron microscopy and gel exclusion chromatography. The addition of the detergent β-octyl glucoside disassembles pili into soluble pilin dimers (19, 41). Crystallization conditions (19), which are exquisitely sensitive to solubility and surface charge changes of the type expected with sequence variation, were unchanged from one batch of pilin to another, suggesting that our experimental conditions have selected for homologous pilin with minimal sequence variation.

Antibody production. Peptide homologs of the MS11 pilin sequence (11) were synthesized as described previously (35) by the solid-phase method (16) with an automated peptide synthesizer. Crude peptides were purified to >90% homogeneity by reverse-phase high-performance liquid chromatography. To ameliorate the possible bias in the results due to the cross-linking protocol itself, two carrier proteins were used with two coupling mechanisms. Peptides were coupled as described by Tainer et al. (35) to the immunogenic carrier protein keyhole limpet hemocyanin through a natural or additional C-terminal cysteine of the peptide by using *N*-maleimidobenzoyl-*N*-hydroxysuccinimide ester as the coupling reagent and to tetanus toxoid through lysine side chains by using glutaraldehyde. Results for antibodies raised against peptides coupled either way were very similar. We have presented both for two examples in Fig. 1 but in general have chosen the higher-titer antisera. Antipeptide antibodies were made in two chronologically separated batches. The first set of antibodies included those raised against residues 1 to 12 (1–12), 13–31, and 115–127. These were coupled only to keyhole limpet hemocyanin. All other peptides were coupled to both carriers. There were two main considerations in choosing peptide breaks. First, the number of tryptophans was minimized, because at the time these peptides were made, tryptophan increased the difficulty of synthesis. This is the reason for the 1-residue breaks at 109 and 119. Second, peptides were chosen to be as long as possible but not longer than 20 residues (9). This size was chosen to optimize the trade-off between sufficient length for at least one antigenic site and minimal loss of resolution.

Two rabbits were immunized with each peptide in a course of three subcutaneous injections of 100 μg of carrier-coupled peptide in complete Freund's adjuvant (1:1) on day 0 and in incomplete Freund's adjuvant (1:1) on days 14 and 21. The rabbits were bled 10 days after the third injection. The rabbits received a booster immunization of an additional injection of 100 μg of carrier-coupled peptide in incomplete Freund's adjuvant 2 weeks after the third injection and were bled again 7 and 14 days later. Two rabbits were injected with unmodified pilin protein according to the same protocol. Two additional rabbits were injected with pilin protein that had been run on a denaturing polyacrylamide gel and electroeluted from a single band of pure pilin. The immunoglobulin fraction of each rabbit serum was separated by dextran sulfate precipitation to remove lipoprotein; this was followed by precipitation with an equal volume of a saturated ammonium sulfate solution (15). The pellet from the ammonium sulfate cut was dialyzed into phosphate-buffered saline (PBS; 10 mM sodium phosphate, 150 mM NaCl, 0.02% NaN_3, pH 7.5). As expected, measured titers varied greatly, from no dilution to a 1:1,600 dilution required to achieve a linear response in an enzyme-linked immunosorbent assay (ELISA) against pili.

As a control, peptide mapping of the antibody specificities of a subset of eight of the antipeptide antibodies was done to verify that they recognized the pilin sequences corresponding to the original peptide antigen (data not shown). Synthetic peptides, which represent the entire MS11 pilin sequence, were manually synthesized on polypropylene pins arranged in a 96-well ELISA plate format (Cambridge Research Biochemicals). The entire protein sequence was represented in overlapping octamers beginning at the amino terminus of the protein and offset by one amino acid at each peptide until the carboxy terminus was reached. These pins were used in a capture ELISA to identify the epitope recognition of antibodies.

The rabbit antipilus sera used in peptide mapping experiments were generated by intramuscular immunization of 100 μg of purified pilin emulsified in an equal volume of Freund's complete adjuvant, followed by a second intramuscular injection with 100 μg of pilin 26 days later with Freund's incomplete adjuvant. The rabbits were bled 5 days after the second injection, and the sera were collected and stored at −20°C.

Human sera from placebo and vaccine-immunized volunteers (1) and from challenged volunteers (33), as well as mouse monoclonal antibodies, were generously donated by the Walter Reed Army Institute of Research. Monoclonal antibodies were prepared against partially purified MS11 pili. Mice were given one primary immunization and two booster immunizations of 0.2 mg of partially purified MS11 pili. Spleen cells from immunized mice were fused to P3X63-Ag8.653 murine myeloma cells and subcloned according to standard procedures. Tissue culture supernatants were screened for reactivity against MS11 pilin on days 10 to 14 after the fusion.

Western blots. Antibodies were characterized by Western blot (immunoblot) analysis (36). Following electrophoresis of pilin on 15% polyacrylamide gels under denaturing conditions (the samples were boiled in the presence of 2% sodium dodecyl sulfate and 5% 2-mercaptoethanol), the protein was transferred to a nitrocellulose filter and incubated with antipeptide antisera. Antibody reactivity was detected by incubation with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (14) and development with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Promega).

Immunoassays. ELISAs were done according to the procedure described by Tainer et al. (35). Polystyrene microtiter wells were coated with 5 pmol of pilin in either PBS, pH 7.5, for bundled pili or 10 mM borate, pH 9.5, for purified single fibers and dried overnight at 37°C. The pili were fixed by washing the plates with methanol. The pili were thus probably partially denatured. This was done to maximize accessibility of all sites on the protein. To minimize variation in pilin sequences, all of the ELISAs were done with a single preparation of pilin. The same reagents were used in ELISAs without drying or in the presence of β-octyl glucoside to completely disassemble fibers into dimers, with similar results but a lower signal-to-noise ratio (data not shown). The wells were blocked with 5% nonfat dry milk (13) in PBS, pH 7.5, or in borate, pH 9.5, as appropriate. Serial dilutions of antibodies in PBS were incubated in the blocked wells. Antibody binding was measured by an enzymatic assay whose final product absorbs light at 400 nm (20). The titer was defined as the antibody concentration that produced half-maximal A_{400}.

For competitive ELISAs, 5 pmol of purified fibers in 50 mM CHES, pH 9.5, was dried on microtiter plates, and wells were blocked as described above. Antibodies diluted to their titer points (determined by direct ELISA against pili in the corresponding buffer) were preincubated at 4°C overnight with serial dilutions (80 to 1.25 pmol) of pilus fibers in PBS. The antibody-pilus mixture was then transferred to the wells containing dried single fibers, and binding of antibodies to the plate-bound pili was measured as described above.

Electron microscopy. For negative staining of samples for electron microscopy, a 3-μl sample of a protein solution (0.001 to 0.1 mg/ml) or a suspension of whole cells was applied to nitrocellulose (Parlodion)-coated 400-mesh grids. Alternatively, the grids were floated facedown on a 60-μl droplet of sample; this was followed by slow horizontal withdrawal. The grids were stained with 1% uranyl formate for 30 s. Micrographs were recorded on Kodak 4489 film with a Hitachi HU12A microscope operating at 75 kV with magnifications between ×12,000 and ×60,000.

Both primary and secondary gold-labeled antibodies were used for immunoelectron microscopy. For primary labeling, purified antipeptide and antiprotein antibodies were gold labeled by using preparations of 4- or 10-nm-diameter colloidal gold label (Janssen Pharmaceutica) and standard techniques (8). Gold-labeled immunoglobulin G probes were stabilized in 1% bovine serum albumin (BSA; Sigma) and stored (for less than 1 week) with 0.02% sodium azide at 5°C. For secondary labeling, goat anti-rabbit immunoglobulin G was purchased from Janssen Pharmaceutica. Protein samples (0.5 mg/ml) were applied to nitrocellulose-coated 400-mesh grids and incubated with primary

unlabeled or gold-labeled antibodies (0.5 to 100 μg in 20 to 200 μl of TBS) for 1 h at 37°C or room temperature. Unlabeled primary antibodies were monitored by incubation for 30 to 60 min with gold-labeled goat anti-rabbit immunoglobulin G (diluted 1/25 to 1/50 in 0.1% BSA in TBS). The grids were blocked before each antibody-labeling step by incubation with 0.3% BSA in TBS. After incubation with gold-labeled antibodies, the grids were washed and stained with 1% uranyl formate. Micrographs were recorded on Kodak 4489 film with a Hitachi HU12A microscope operating at 75 kV with a magnification of ×30,000. An average of seven micrographs were examined for each antibody-pilus interaction.

Peptide mapping of antibody specificities. For peptide scanning of rabbit polyclonal and mouse monoclonal ascites fluids, the set of all overlapping hexameric peptides from the MS11 pilin sequence was synthesized on polyethylene rods by using the *t*-butyloxycarbonyl and 9-fluoro-enyl-methyl-oxy-carbonyl strategies (10). Mouse monoclonal antibodies were also tested against comprehensive sets of overlapping octameric peptides, synthesized as immobilized biotin-conjugated peptides with the structure biotin-Ser-Gly-Ser-Gly-peptide (24, 42). For peptide scanning of human antibody responses to an infection challenge with *N. gonorrhoeae* MS11, the same pin-bound strategy was used with overlapping octameric peptides. Pilin from the challenge strain was resequenced and found to contain two amino acid changes at positions 144 and 158 (33). For mapping of human antibody response to the closely related Pgh 3-2 pilin vaccine and subsequent infections (1), comprehensive sets of overlapping octameric peptides were synthesized as immobilized biotin-conjugated peptides with the structure biotin-Ser-Gly-Ser-Gly-peptide (24, 42). Pgh 3-2 and MS11 sequence differences are found in peptides 79–93 (six differences), 94–108 (four differences), 110–120 (two differences), 122–139 (eight differences), and 140–159 (three differences).

At each dilution of a given antiserum, the test background was obtained from the nonreacting peptides (10). Analysis of the corrected extinctions showed that they were linearly dependent on the concentration of the antiserum used in the test. Titers were estimated from the data to be the reciprocal of the dilution of the antiserum that would give an extinction of 1.00 above the test background. Titers less than 200 (twice the slope of the background) were ignored, and those greater than 2,000 were not further resolved. Conjugated peptides (10 to 20 per carrier molecule) were adjusted to 50 pmol per well of microtiter plates, and reactivity was measured by ELISA.

RESULTS

A comprehensive set of antipeptide antibodies for pilin. A set of polyclonal antisera was raised against 11 synthetic peptides representing the entire sequence of the pilin protein from *N. gonorrhoeae* MS11. The peptides covered residues 1–12, 13–31, 18–36, 37–56, 58–78, 79–93, 94–108, 110–120, 115–127, 122–129, and 140–159 of the 159-residue protein. The synthetic peptides were chosen to average 15 residues in length. This is long enough to increase the potential for recognition of the intact protein by the antipeptide antibodies, despite possible variations between the antigenic surfaces of the peptide and the protein (9). Longer peptides were avoided to minimize the likelihood of nonnative stable peptide conformations and loss of resolution in location of sequence regions. Antipeptide antibodies were used in competitive ELISAs and immunoelectron microscopy to identify accessible and buried regions of the pilin sequence in purified pilus fibers obtained by repeated solubilization and precipitation steps by manipulation of salt and pH conditions (see Materials and Methods). Each antipeptide antibody detected pilin on Western blots (data not shown).

Mapping residues buried in the pilus fiber by competitive ELISA. To determine the accessibility of each peptide in pilus fibers, competitive ELISAs were used to assay the relative binding of each individual antipeptide antibody to native, bundled pilus fibers versus denatured fibers on plates. Titers for the reactivity of each antipeptide antibody with pilin and with its peptide were determined by ELISA. Because the 11 synthetic peptides used to generate peptide-specific sera were chosen from the entire pilin primary sequence rather than the most immunogenic regions, reactivities of the antipeptide antibodies varied over a wide range. To adjust for this, binding in competitive ELISAs was always evaluated at the titer point of each individual antipeptide antibody. (The antipeptide serum against residues 1–12 does not recognize native pilin in ELISA experiments and recognizes denatured pilin only weakly on a Western blot, so results from this antibody are not presented.) Data from these experiments divided the binding of antipeptide antibodies into four general classes: (i) no fiber competition, (ii) weak competition, (iii) good fiber competition, and (iv) very strong competition such that the control binding level is never regained. Binding to pili and denatured pili dried on plates was competitively inhibited by bundled pilus fibers in solution for all antisera except those directed to pilin residues 13–31 and 18–36, indicating that these residues are largely inaccessible to antibody in the context of the intact fiber. Binding of antibodies against pilin peptides 79–93 (Fig. 1) and 94–108 was inhibited weakly by assembled fibers. For pilin peptide antibodies against residues 37–56 (Fig. 1A), 110–120 (Fig. 1B), and 115–127, fibers competitively inhibited binding to plate-bound pilin, indicating that these sequences were exposed in whole or in part on the sides and/or ends of the fiber.

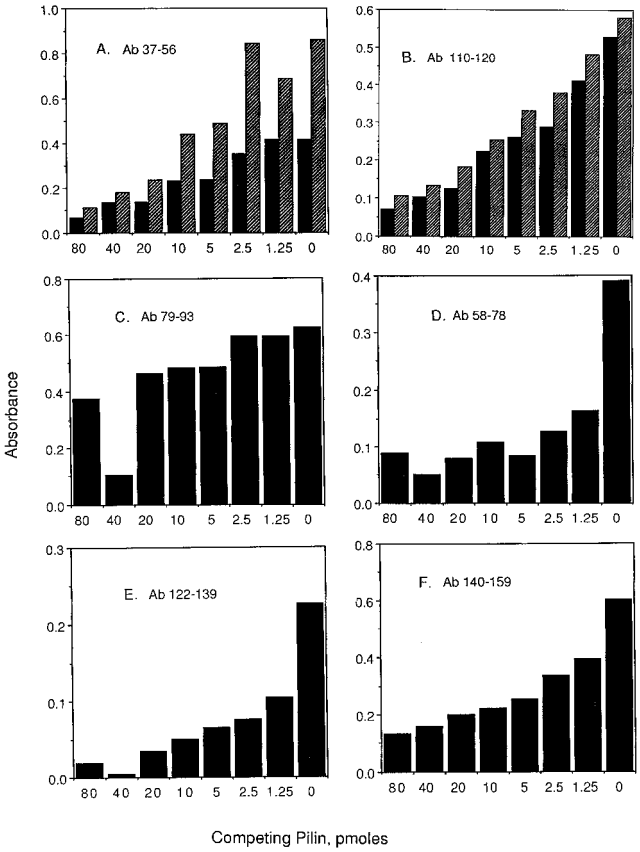

FIG. 1. Competitive binding of antibodies to pili. Antipeptide antibodies were preincubated with various amounts of pilus fibers in solution and then exposed to pili dried onto ELISA plates. All graphs show the A_{400} (*y* axis) (see Materials and Methods), indicating the relative number of antibodies bound to the ELISA plate versus picomoles of competing pilin, in the form of assembled fibers. The peptide used to elicit the antibody (Ab) response is given for each graph. Similar results were obtained for independent experimental runs and for antibodies raised to keyhole limpet hemocyanin- or tetanus toxoid-coupled peptides as shown, for example, in panels A and B (hatched bars, keyhole limpet hemocyanin; solid bars, tetanus toxoid). Fibers competed for binding of antibodies raised against denatured whole pilin or pilus fibers. The sequence of MS11 pilin used for peptide synthesis and peptide mapping (Fig. 3) in all of the following examples is as follows:

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  1 FTLIELMIVI AIVGILAAVA LPAYQDYTAR AQVSEAILLA EGQKSAVTEY
 51 YLNHGKWPEN NTSAGVASPP SDIKGKYVKE VEVKNGVVTA TMLSSGVNNE
101 IKGKKLSLWA RRENGSVKWF CGQPVTRTDD DTVADAKDGK EIDTKHLPST
151 CRDKASDAK
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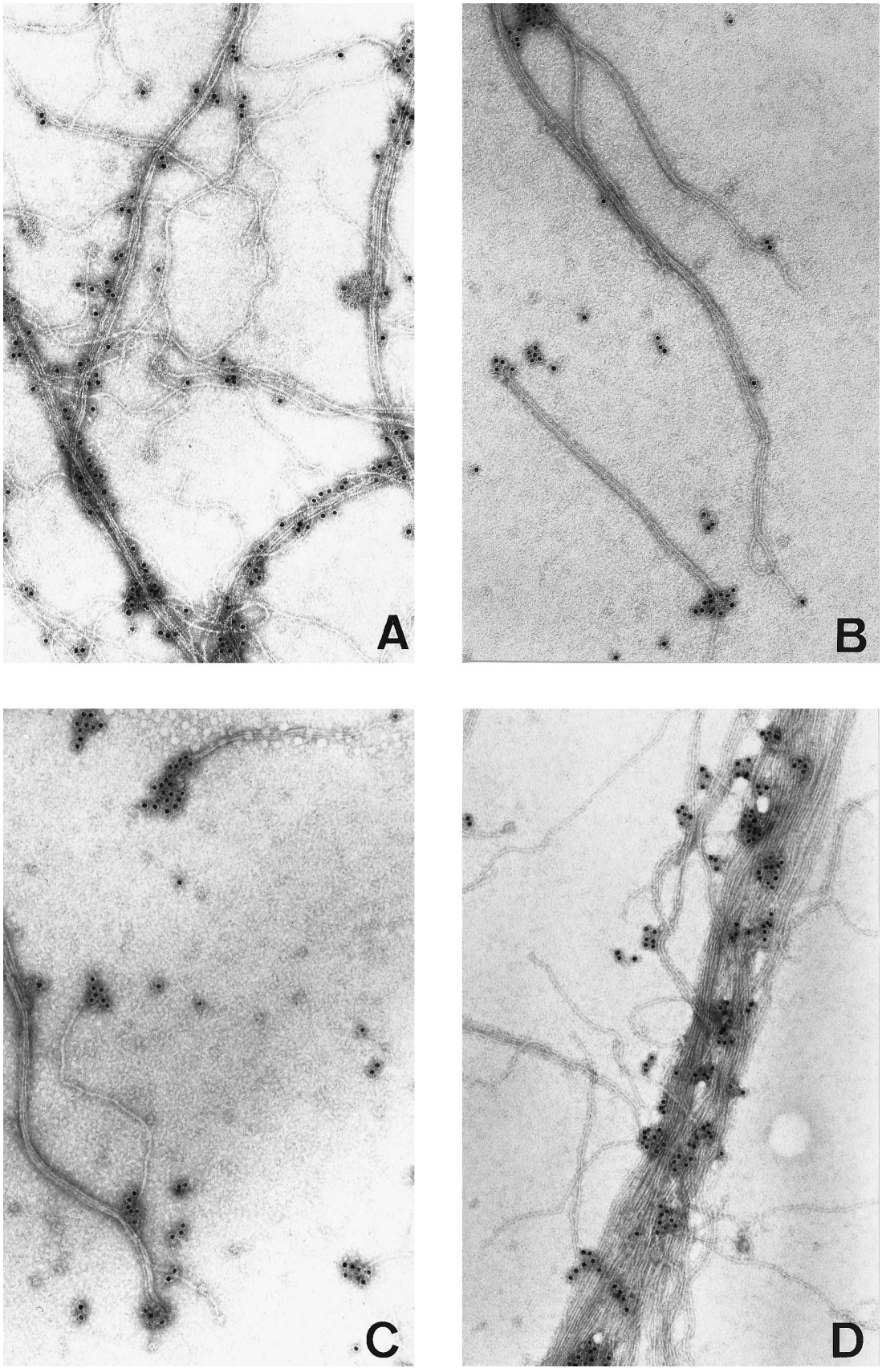

FIG. 2. Immunoelectron micrographs of gold-labeled rabbit antipilin and antipeptide antibodies bound to purified pili. (A) Antipilin antibodies bound the entire length of the pilus fibers. (B) Antipeptide antibodies to pilin sequence 37–56 show a strong preference for binding ends. (C) Antipeptide antibodies to pilin sequence 94–108 also show a strong preference for binding ends. (D) Antipeptide antibodies to pilin sequence 110–120 bind to ends and sides of fibers. All micrographs show no significant background in controls without primary antibodies; therefore, existing background apparently represents pilin subunits or other small pilin aggregates. For size reference, each pilus fiber is about 60 Å (6.0 nm) in diameter. (A, B, and D) Micrographs were recorded at a magnification of ×30,000. (C) Micrograph was recorded at a magnification of ×42,000.

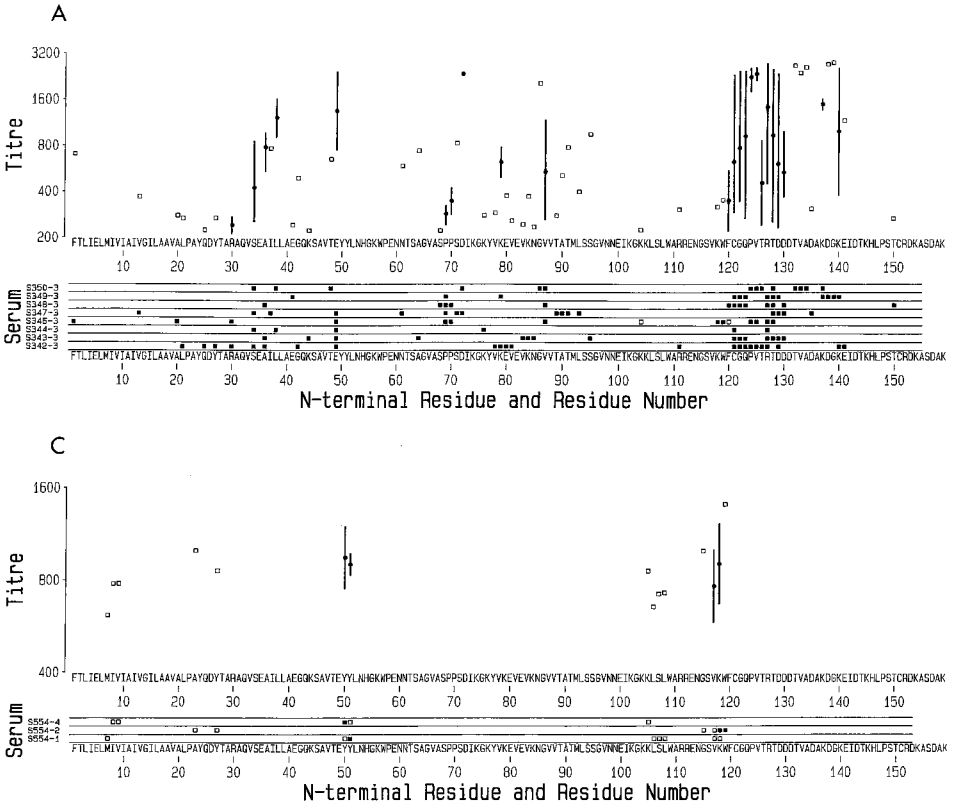

FIG. 3. Peptide mapping of antipilus serum specificities. Refer to the legend to Fig. 1 for the sequence of the MS11 pilin used for the synthesis of all peptides used in mapping. (A) Rabbit polyclonal antipilus sera, diluted 1/200, mapped against hexameric pin-bound peptides, numbered by the position of their N-terminal residue within the *N. gonorrhoeae* MS11 pilin sequence. The top panel shows the individual titer (square) or, when more than one serum reacted, the geometric mean titer (circle) and range of titers (vertical bar) of the antisera. The bottom panel summarizes how each of the eight sera reacted individually. Filled squares represent signals greater than 3 standard deviations above background, and empty squares represent signals 2 to 3 standard deviations above background. (B) Two high-titer mouse ascites fluid samples diluted 1/3,000, mapped against pin-bound hexameric peptides. The top panel is ascites no. 6-96-6. The bottom panel is ascites no. 6-526-12. An additional high-titer monoclonal antibody 6-608-5 recognizes peptide 117–126 (data not shown here but summarized in Fig. 3). (C) Sera from human volunteers challenged with *N. gonorrhoeae* MS11, diluted 1/2,000, are mapped against pin-bound octameric peptides numbered by the position of their N-terminal residue within the pilin sequence. The top panel shows the individual titer (square) or, when more than one serum reacted, the geometric mean titer (circle) and range of titers (vertical bar) of the antisera. The bottom panel summarizes how each of the three sera reacted individually. Filled squares represent signals greater than 3 standard deviations above background, and empty squares represent signals 2 to 3 standard deviations above background. (D) Sera from a human parenteral vaccine trial (1) mapped against immobilized biotinylated octameric peptides. Volunteers were immunized with either a placebo or pilin from the *N. gonorrhoeae* strain Pgh 3-2. The top panel is a pool of nine sera from the uninfected vaccine group. The middle panel is a pool of six sera from the placebo group from volunteers who subsequently presented with gonorrhea, and the bottom panel is a pool of four sera from the vaccine group from volunteers who subsequently presented with gonorrhea. Each serum is diluted 1/200. Signals were not differentiated above an ELISA absorbance of 2.0.

Fibers competitively inhibit binding so well that the control binding level is never regained for pilin antibodies against residues 58–78 (Fig. 1D), 122–139 (Fig. 1E), and 140–159 (Fig. 1F), indicating high accessibility of these sequences or increased affinity of antipeptide antibody binding in the fiber environment. Two of these peptides are within the hypervariable region responsible for antigenic variation of pilin and, therefore, are expected to be highly accessible on the intact fiber.

Defining exposure in the pilus fiber by immunoelectron microscopy with antipeptide antibodies. Immunoelectron microscopy with antipilus and antipeptide antibodies was done on pili attached to whole cells and on purified pilus fibers (see Materials and Methods). These immunoelectron micrographs are consistent with the specific information on surface-exposed and buried sequence regions in pilus fibers provided by competitive ELISAs. Antipeptide antibodies directed to pilin sequences were divisible into three general classes as illustrated by the representative data illustrated in Fig. 2: (i) negligible binding to fibers, (ii) binding to both sides and ends, and (iii) strong preference for binding pilus ends. Thus, some antipeptide antibodies showed binding similar to that of antibodies against the whole pilin protein (Fig. 2A); they bound along the sides of pili on cells and purified pili, as well as at their ends. We have not attempted to determine the polarity of the purified fibers with these antibodies. The antipeptide antibodies with negligible binding to fibers, those against peptides 13–31 and 18–36, are the same ones with no measurable competition in ELISA experiments. Antibodies against peptides homologous to pilin residues 37–56 and 94–108 bound primarily at the end of the pilus, suggesting that these regions were mostly buried in the longitudinal assembly of the pilus (Fig. 2B and C).

Antibodies against peptides representing pilin residues 58–78, 79–93, 110–120, 115–127, 122–139, and 140–159 bound along the sides in addition to the ends of the pili, indicating that these sequences were surface-exposed regions along the assembled fiber (Fig. 2D). Binding to the sides of fibers led to aggregation of pilus fibers. As the antibody concentration was increased, bundles of pilus fibers were observed (Fig. 2D),

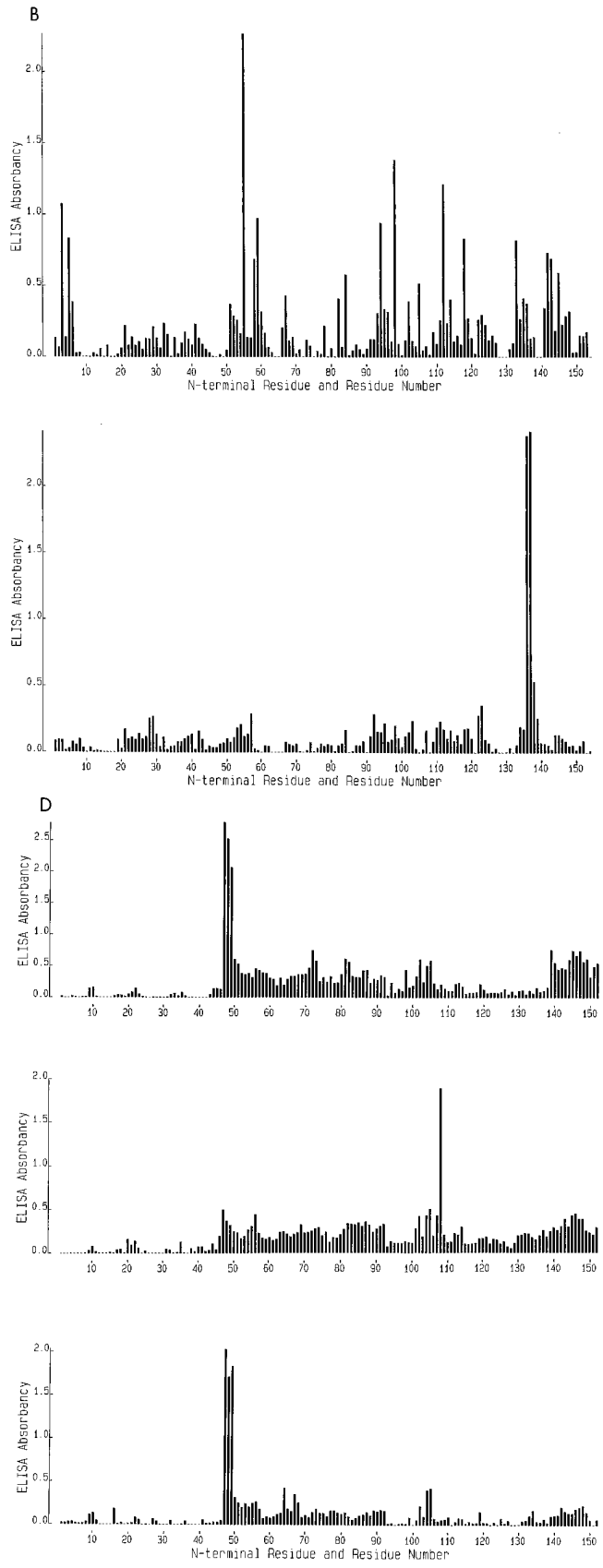

FIG. 3—*Continued.*

presumably because of cross-linking of the fibers by the divalent antibodies. Pairs of antibodies against the same peptide sequence coupled to different carriers (tetanus toxoid or keyhole limpet hemocyanin) bound pilus fibers in the same locations (data not shown).

Peptide mapping of antipilus serum specificities from three species. Peptide mapping of antiserum specificities was used to further examine pilus immunogenicity and assembly for rabbit polyclonal, mouse monoclonal, and human antisera. Determination of the conservation of responses among these species is relevant, because immunogenicity studies often rely on the experimentally accessible mouse or rabbit while humans are the only animals that can contract or carry gonorrhea.

Polyclonal rabbit antipilus sera (Fig. 3A), monoclonal mouse antipilus sera (Fig. 3B), sera from human volunteers challenged with *N. gonorrhoeae* MS11 (Fig. 3C), and sera from volunteers vaccinated with strain Pgh 3-2 pilin (Fig. 3D) were used to identify and compare immune responses in these three species. The integration of results from fiber competition ELISA, immunoelectron microscopy, and peptide mapping of antibody specificities (Fig. 4) provided a basis for assessing the related properties of accessibility, antigenicity, immunogenicity, and cross-species response.

More than half of the eight rabbit sera reacted with the pin-bound hexameric peptide 49–54 and with a group of peptides beginning with residues 121 to 128 in the hypervariable region of the MS11 sequence. Four of the sera also reacted with peptide 34–39, peptide 69–74, and peptide 129–134 and with peptides whose sequences are completely overlapped by other peptides in the hypervariable region each of which reacts with five or six sera. The highest titer responses (titers greater than 2,400 with a signal-to-noise ratio better than 3 standard deviations above the background) were to peptides 49–54 and 72–77 and to overlapping peptides which cover residues 121–145.

Three high-titer mouse monoclonal antibodies mapped against pin-bound hexameric peptides show that the strongest responses are against hexapeptides or octapeptides beginning with residues 55, 117 to 119, and 136 to 137 (Fig. 3B). Of the three human volunteers challenged with MS11, sera reacted at a level 3 or more standard deviations above background measurements for four octapeptides, starting at residues 50, 51, 118, and 119 (Fig. 3C). These results suggest that pilin regions 50–58 and 118–126 are exposed in vivo during gonococcal infection. For sera from a gonococcal vaccine trial in which volunteers were vaccinated with either a placebo or pilin from *N. gonorrhoeae* Pgh 3-2 (1), significant peaks were selectively enhanced by pooling several sera before peptide mapping (Fig. 3D). Human sera from vaccinated volunteers reacted with peptides covering residues 47–56, whether or not these volunteers subsequently presented with gonorrhea. Volunteers who received only a placebo and later presented with gonorrhea appeared to contain antipilus antibodies to the peptide 108–115. Human volunteers vaccinated with pilin or volunteers challenged with gonococci each had clear responses to octameric peptides beginning with MS11 pilin residues 47 to 51.

DISCUSSION

When combined with sequence data for various gonococcal pilin variants, the immunological and electron microscopy data presented here (Fig. 4) suggest a model for the structural assembly of pilin into the pilus fiber. Among different gonococcal pilin variants, the N-terminal third (residues 1–53) is highly sequence conserved, whereas the remainder of the molecule undergoes antigenic variation (11). The competitive

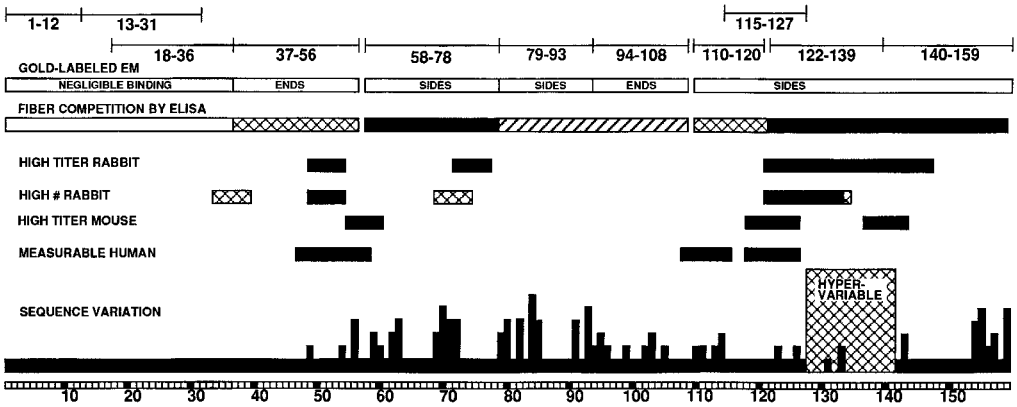

FIG. 4. Antibody reactivity mapped on the pilin sequence. This figure summarizes the data on pilin antigenicity and assembly presented throughout the paper and shows the peptides used as antigens along the top. Binding of reassembled fibers by gold-labeled antipeptide antibodies is shown in the second line. For fiber competition (third line), dark shading indicates strong competition which does not return to the control level, cross-hatching indicates strong competition, hatching indicates weak competition, and no shading or hatching indicates no competition. For rabbit, mouse, and human antiserum specificity mapping (lines 4 to 7) high-titer or large number of responses to a given peptide is indicated by dark shading, and fewer but significant responses (see the text and Fig. 3) are indicated by cross-hatching. Finally, the sequence variation among sequenced *N. gonorrhoeae* pilins is represented in the bottom bar graph, with the number of variations in the sequence with respect to MS11 pilin at a given position indicated by the height of the bar. For positions outside the hypervariable region as well as at conserved residues 131 and 133 within the hypervariable region, each insertion, deletion, or single amino acid change is counted once. The hypervariable region (128–141), in which multiple insertions, deletions, and amino acid substitutions are accommodated, is indicated. The alignment includes 78 pilin variants from eight strains of *N. gonorrhoeae* (11, 18, 30, 32–34). EM, electron microscopy.

ELISA data suggest that within this conserved region, the initial hydrophobic sequence (residues 1–36) is predominantly buried by lateral assembly, the side-by-side packing of pilin protein subunits across the thickness of a pilus fiber. Furthermore, this buried region is not significantly immunogenic in any of the species examined. The more polar remainder of the sequence-conserved N-terminal region (residues 37–56) appears to be located at the end of the pilus fiber, where it contributes to longitudinal assembly. Thus, the entire N-terminal one-third of the pilin protein apparently codes for pilus assembly; this explains its extreme sequence conservation from strain to strain. The immunoelectron microscopy results are in good agreement with the competitive ELISA data. For example, the three antipeptide antibodies for which fibers compete most strongly are side binders, consistent with the greater number of side sites over end sites in assembled fibers.

The localized site covering residues 49–56 is especially noteworthy. Our antipeptide antibody raised against residues 37–56 binds at the ends of fibers, and its binding to pilin protein is inhibited by fibers. Human volunteers vaccinated with pilin or volunteers challenged with *N. gonorrhoeae* each had clear responses to octameric peptides beginning with MS11 pilin residues 47 to 51. The region 50–57 was immunogenic in an MS11 bacterial challenge study and in Pgh 3-2 pilin-vaccinated volunteers who later presented with gonorrhea, suggesting that it may be at least partly exposed on bacteria in vivo. These data on sites exposed to the human immune response during infection are important because of the possible bundling, shearing, and denaturation of fibers used as immunogens in murine or rabbit studies. This 49–54 region also elicits high-titer rabbit polyclonal sera and is immunogenic in mice (Fig. 4). Furthermore, it encompasses the antigenic determinant from positions 48 to 60 that is cross-reactive for multiple gonococcal strains (22). The monoclonal antibody SM1, which reacts with all *Neisseria* type IV pilins tested, recognizes this epitope, with the minimum reacting peptide being residues 49–53 (39). The conserved sequence 41–50 has also been identified as important for the attachment of gonococci to endocervical cells (23). Thus, we propose that this conserved sequence (residues 49–56) participates in the protein subunit contacts leading to longitudinal fiber assembly and is at least partially exposed on the end of the pilus fiber. This packing arrangement produces a conserved cell-binding site on the pilus with minimal exposure to the immune system, which can function in cell attachment even if pili have been sheared or partially disassembled. Such attachment could be mediated indirectly; for example, either the pilin region 49–56 or 94–108 could function as the binding site on pili for the accessory protein PilC (25). Although the antipeptide antibody against sequence residues 94–108 also binds fiber ends, it appears less exposed by competitive ELISA and is not highly immunogenic.

Antibodies against peptides from the variable C-terminal third of pilin bind to the sides of pilin fibers, and their binding is competitively inhibited by fibers. This part of the sequence, contained in the third cyanogen bromide cleavage fragment of pilin (residues 93–159), is known to be immunodominant (27), and accordingly, the antigenic response from all three species tested is high in this fragment and probably involves multiple sites. However, the response to this region is not as tightly localized as the response to residues 49–56, probably because of the extent of sequence variation in the C-terminal third of pilin, consistent with the results of Rothbard et al. (22), who defined residues 121–134 and 135–151 as two separate epitopes within the disulfide loop. Our peptide mapping data corroborate that antigenic sites in the final third of pilin are probably made up of a nonlinear set of amino acid residues, as there is not a sharply defined peptide response but rather a broad spread of reacting peptides there. Yet, there is a highly reactive site common to all three species, a "hot spot" at residues 121–126. Peptides encompassing this region are accessible in assembled fibers and compete strongly with fibers for antibody binding. This conserved site is also the most reactive peptide for monoclonal antibody SM2 (39), which reacts to some extent with a range of peptides covering residues 108–127. So, within the generally immunogenic and variable C-terminal third of pilin, there is a localized hot spot showcased by our data.

While the first third of pilin contains the end-exposed reac-

tive site around residue 50 and the final third of pilin contains the broadly reactive hypervariable region as well as an antigenic hot spot at residues 121–126, the middle third of the pilin sequence does not seem to contain any highly reactive, strongly competing regions. The conserved regions seen in the C-terminal two-thirds of the sequence primarily reflect constraints imposed by functional requirements distinct from the assembly, which appear to allow more freedom for sequence variation. This assembly model and the N-terminal sequence conservation suggest a mechanism for incorporation of pilin into the pilus fiber: sequence homology with the conserved pilin subunit interface could permit assembly into the fiber, while allowing extensive sequence variation and, hence, functional differentiation elsewhere.

Our results highlight the problems involved in making peptide vaccines against self-assembling proteins but also provide useful information on cross-species response and the accessibility of semiconserved regions to the immune response. If the most sequence-conserved regions of the protein are buried by assembly of the fiber, then antibodies directed against these sequences will not recognize the intact fiber on the whole organism and therefore will not be able to inhibit attachment. One such sequence-conserved pilin peptide (residues 21–35) generated an antibody response but did not block attachment of gonococci to endocervical cells (22). Our data suggest that this sequence is buried in lateral fiber assembly and is thus unavailable for binding in the assembled fiber. The variation in immune response among species should also be considered when using results from rabbits, mice, or other animals to guide vaccine design for humans. Our peptide mapping experiments reveal some variation in immune response among these species, while only two general sequence regions (near residues 49 and 121) consistently show strong antigenicity. On the basis of this work, the two most promising regions for inclusion in multicomponent vaccine design would be the semiconserved regions 49–56, identified here as participating in the longitudinal fiber assembly, and 121–126, shown here to be exposed on fiber sides. The conservation within two exposed and immunogenic regions suggests that their sequence variation is limited by restraints imposed by their role in assembly, folding, or functional interactions. As both of these regions are immunogenic in three species, a combination of peptides (15 to 20 amino acids long) representing known variants of these two regions may be worth testing for the potential to raise cross-reactive responses of potential therapeutic value. Additionally, the semiconserved region 58–78 should be considered for inclusion, as it is immunogenic when presented as a peptide conjugate in rabbits and competes very strongly for fiber binding.

The site-directed antibody studies presented here provide specific information on the assembly of pilin protein into the pilus fiber. The peptide mapping of antibody specificities in rabbit, mouse, and human antisera defines pilus regions exposed to immune recognition. The two sites identified from the MS11 challenge antisera (50–58 and 118–126) reveal regions exposed on infectious bacteria in an intraurethral challenge. Likely due to the extreme antigenic variation of pilin during infection, the human antisera do not recognize MS11 peptides in the C-terminal hypervariable sequence regions (residues 128–141), revealing the limitations of this experiment. This approach and these results provide a view of bacterial pilus assembly that is complementary to that of electron microscopy image reconstruction, such as has recently been done with *Escherichia coli* PapA pilin (4) and X-ray crystallographic studies of the pilin subunit in progress (19). Image reconstruction can yield a model for pilin packing into the fiber and X-ray crystallography of the subunit fold, but these methods do not identify sequence-exposed and buried regions within the fiber as do the antibody mapping results. These immunological data will be useful for relating the crystallographic structure of the pilin subunit (19, 19a) to the assembled pilus fiber and for testing the eventual design of peptides that might provide a neutralizing antibody response. Our results also provide a comprehensive database for the design of new antipeptide antibodies to provide greater resolution in the definition of buried and exposed regions of the pilin sequence within the assembled pilus fiber.

ACKNOWLEDGMENTS

We thank Richard Houghten, The Scripps Research Institute, and Johnson and Johnson Biotechnology, Inc., San Diego, Calif., for peptide synthesis; Michael Capozza, Kelly Slater, and Bradley Kays for growing and harvesting *N. gonorrhoeae*; and the Walter Reed Army Institute of Research for the gift of human sera.

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Editor: B. I. Eisenstein