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Short communication

Reactivity of *Haemophilus influenzae* type b anti-pili antibodies

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The reactivity of anti-pilus antibodies to native and denatured *Haemophilus influenzae* b (Hib) pili was studied using rabbit serum prepared against piliated *H. influenzae* b strain M43 (p+) and adsorbed with its non-piliated variant, strain M42 (p−). The specificity of the adsorbed serum for Hib pili was documented by immunogold electron microscopy and by immunoprecipitation, which revealed the 24 kDa pilin band from strain M43 (p+) that was not seen on strain M42 (p−). In immunodot assays, the anti-pilus antibodies reacted with the native pili present on the outer membrane of strain M43 (p+), but on Western blot assay using denatured outer membranes, the anti-pilus antibodies did not react with the 24 kDa pilin subunit. These data demonstrate that the anti-pilus antibodies in the adsorbed serum recognize conformational epitopes that depend on the tertiary or quaternary structure of Hib pili.

Key words: *Haemophilus influenzae* type b; pili; adherence; fimbriae.

Introduction

The initial event in the pathogenesis of infection with *Haemophilus influenzae* b (Hib), a significant pathogen of infants and young children, is colonization of the respiratory mucosa.[1,2] Results of previous studies indicate that Hib, like many other organisms that colonize mucosal surfaces,[3] may attach to respiratory epithelial cells by means of pili.[4–7] However, the precise role of pili in the pathogenesis of Hib infections remains unclear. In addition, although Hib pili appear to be immunogenic[7–9] both the role of anti-pili antibodies in protection against Hib disease, and the spectrum of their immunologic activity, remain unknown.

In this study, the reactivity of polyclonal antiserum that is specific for Hib pili was studied by slide agglutination of whole organisms, by immunodot analysis using native outer membrane proteins of Hib, and by Western blot analysis using denatured outer membrane proteins of Hib.

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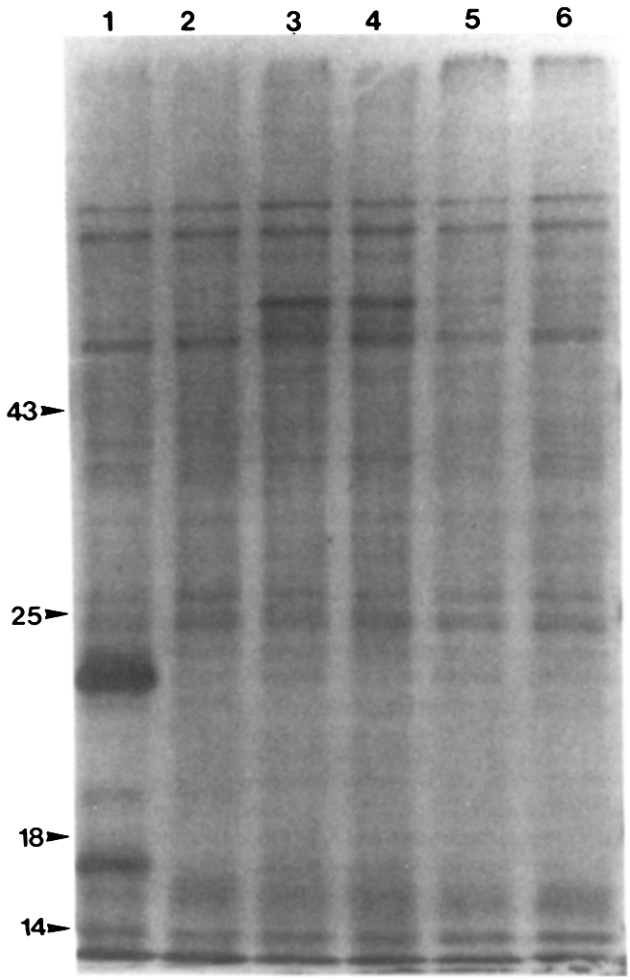

Fig. 1. Radioimmunoprecipitation of *H. influenzae* type b strains M43(p+) (lanes 1,3,5) and M42(p−) (lanes 2,4,6) by adsorbed antiserum (lanes 1 and 2), normal rabbit serum (lanes 3 and 4), and no serum (lanes 5 and 6). Molecular weight markers are indicated on the left.

Results and discussion

Rabbit antiserum raised against whole, live Hib strain M43p(+) agglutinated strain M43p(+) as well as strain M42p(−), a non-piliated variant of M43p+. Upon adsorbing the serum with M42(p−), however, only the piliated strain was agglutinated, suggesting that the antibodies in the adsorbed antiserum were specific for pili. Non-immune rabbit serum did not agglutinate either organism.

To further investigate the specificity of the adsorbed serum, immunoprecipitation of radiolabelled M43(p+) and M42(p−) antigens was performed. Antibodies from the adsorbed antisera immunoprecipitated the 24 kDa pilin (the major structural subunit of pili) from strain M43(p+) (Fig. 1) but not from M42(p−). In addition, the adsorbed serum also precipitated a 17 kDa protein from M43(p+) but not from M42(p−). The identity of this protein is unknown, but it may represent a minor structural protein of pili, or possibly an adhesin protein.[10–12] The polypeptides of strain M43(p+) precipitated by normal rabbit serum did not differ from those precipitated by M42(p−).

Additional evidence for the pilus specificity of the adsorbed antiserum was obtained by using immunogold electron microscopy. Antibodies from the adsorbed immune

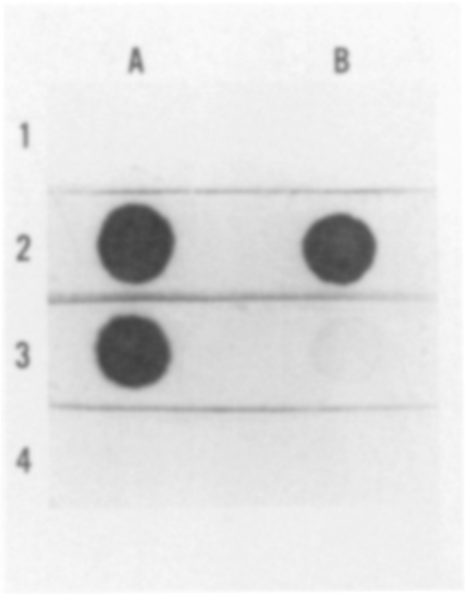

Fig. 2. Immunodot analysis of Hib outer membrane preparations. Dots in column A are outer membrane preparations from Hib strain M43(p+) and in column B, from M42(p−). Row 1 was reacted with non-immune rabbit serum; row 2 with immune serum; row 3 with immune serum adsorbed with M42(p−); row 4 with adsorbed rabbit serum further absorbed with M43(p+).

serum were bound to pili on the surface of strain M43(p+) (data not shown), and essentially no gold particles were associated with the cell membranes of either strain M43(p+) or M42(p−). From these data we conclude that the adsorbed antiserum contains antibodies that recognize native Hib pili and does not contain significant amounts of antibodies against other Hib outer membrane antigens.

During studies of the immunogenicity and vaccine efficacy of bacterial fimbrial antigens, several investigators[13–15] have noted the importance of conformational epitopes on bacterial fimbriae or pili. Therefore, we conducted experiments to determine the ability of the antibodies in adsorbed serum to bind to native and denatured pilus antigens.

In immunodot assays using native antigens, the adsorbed serum bound to outer membrane preparations of strain M43(p+) but not to those of strain M42(p−) (Fig. 2). However, in Western blot assays using denatured antigens, the adsorbed serum did not react with the 24 kDa pilin band of strain M43(p+) (Fig. 3). Furthermore, in an immunodot assay using denatured outer membrane proteins, the adsorbed serum did not bind to strain M43(p+) (data not shown).

Thus, the anti-pilus antibodies in the adsorbed antiserum appear to recognize conformational epitopes that depend on tertiary or quaternary structure of pili and are lost on denaturation. Future studies of the immune responses to Hib pili should employ antibody assays that use native antigens, such as whole cell agglutination or colony immunodot analysis, as well as the denatured antigens used in Western blot assay.

Materials and methods

Bacteria. The piliated *H. influenzae* b strain M43(p+), or AO-2, and its non-piliated variant strain M42(p−), or AO-1, have been previously described.[4]

Preparation of outer membrane proteins. Outer membrane proteins (which included pili) of strains M43(p+) and M42(p−) were prepared using the method of Barenkamp *et al.*[16] In brief,

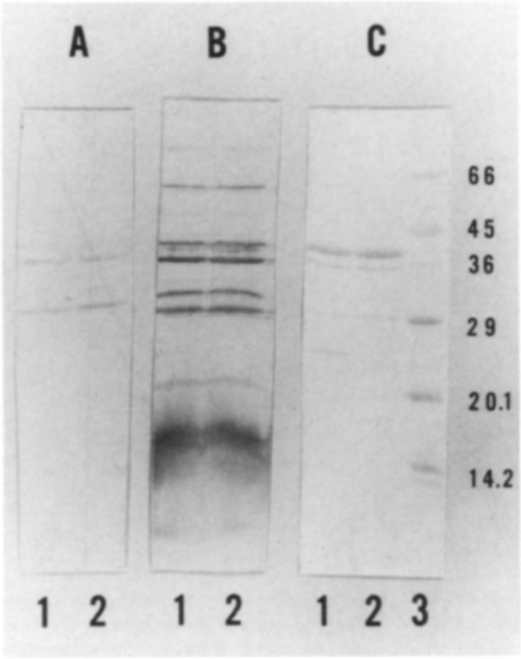

Fig. 3. Western blot analysis of outer membrane proteins of Hib strains M43(p+) and M42(p−). Panels A and B show nitrocellulose membranes containing transblotted proteins of strain M43(p+) in lane 1 and strain M42(p−) in lane 2 that were developed with absorbed serum (Panel A) and with immune serum (Panel B). Antibodies in the adsorbed antiserum recognized polypeptides of approximately 29 kDa and 36 kDa in Western blots, which probably represent small amounts of residual non-pilus antibodies that were not completely removed by adsorption. Panel C shows proteins of Hib strain M43(p+) in lane 1, strain M42(p−) in lane 2, and molecular weight markers in lane 3 electrotransferred from the same gel as Panels A and B to a nitrocellulose membrane and stained with amido black. The presence of the 24 kDa pilin band in lane 1 confirms the adequacy of the electro-transfer.

organisms grown overnight at 37°C on Levinthal agar were suspended in 10 mM HEPES buffer (pH 7.4), and sonicated; debris was removed by differential centrifugation. The membranes were extracted with 2% sodium N-laurylsarcosine and the detergent-insoluble fraction was collected by centrifugation. The total protein concentration of the outer membrane preparation was determined by the Peterson modification of the method of Lowry.[17]

The outer membrane protein preparations were electrophoretically resolved using the sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS–PAGE) method of Laemmli, as modified by Loeb *et al.*[18] Outer membrane preparations containing 12.5 μg protein were diluted in an equal volume of sample buffer and boiled for 5 min. The prepared samples were resolved by electrophoresis and the gels were stained with Coomassie brilliant blue and then destained.[19]

Preparation of unadsorbed immune antiserum. A Pasteurella-free young adult male New Zealand white rabbit was immunized with live *H. influenzae* b strain M43(p+) as follows: Strain M43(p+) was grown on chocolate agar overnight at 37°C. The organisms were suspended in 0.1 M phosphate-buffered saline (pH 7.3) with 0.1% gelatin (PBS-G), and adjusted to the desired concentration. The immunization schedule was a modification of the protocol of Alexander,[20] using an initial inoculum of 1×10^5 colony-forming units (cfu) which was gradually increased over 10 days to a final inoculum of 1×10^6 cfu. The inocula were given intravenously for 3 weeks, followed by a 2 week rest period which constituted one cycle. Subsequent cycles utilized an initial inoculum of 5×10^5 cfu which was increased to 1×10^6 cfu over the next two days. A total of four cycles was given, followed by periodic booster injections of 1×10^6 cfu. The rabbit experienced no apparent adverse reactions to the immunizations.

Preparation of adsorbed antiserum. The immune serum was incubated at room temperature with an equal volume of a dense suspension of strain M42(p−) for 30 min. After removing the bacteria by centrifugation, the serum was further adsorbed with sonicated M42(p−) organisms, and with an outer membrane protein preparation of strain M42(p−).[16] For use in some experiments, the adsorbed antiserum was similarly further adsorbed with outer membrane protein of strain M43(p+) to remove anti-pilus antibodies.

Immunogold electron microscopy. Specimen grids were prepared by application of formvar film, by carbon coating, and by exposure to a glow discharge for 5 min. Five μl of broth culture of strains M43(p+) or M42(p−) were applied to the grid for 1 min. The grids were then rinsed with PBS and floated on a drop of adsorbed antisera (1 : 40) for 15 min. After rinsing again, the grids were floated for 1 h on Protein-A-Gold (15 nm colloidal gold–protein-A complex, Janssen Life Life Sciences, Lamnerdies, Belgium), diluted 1 : 10 in PBS. The grids were then rinsed by gentle agitation in PBS and negatively stained with 0.5% phosphototungstic acid, and examined in a JEOL 100cx electron microscope.

Immunoprecipitation. Specificity of the anti-pilus antibodies in the adsorbed serum was further tested by immunoprecipitation. Hib proteins were radiolabelled by growing overnight in MI medium[21] in which the concentration of methionine, cysteine and K_2SO_4 was reduced to 0.05 mM and 250 μCi [^{35}S]-methionine (1100 Ci/mmole; ICN Biomedicals Inc, Irvine, CA) per ml had been added. Immunoprecipitation assays were done as previously described[22] except protein-A sepharose (Sigma Chemical) was used to precipitate antibody-antigen complexes. The sepharose resin was washed three times with 50 mM Tris–HCl, pH 8.0+15 mM NaCl+0.1 mM EDTA+2% Triton X-100 and three times with 10 mM Tris–HCl, pH 8.0. Radiolabeled proteins were released from the protein-A by adding SDS–PAGE sample buffer and heating to 110°C for 10 min. The resin was removed by brief centrifugation and the supernatant was analyzed by SDS–PAGE.[23] The gel was fixed with water/methanol/acetic acid (50 : 40 : 10), then soaked in En^{3}hance (New England Nuclear, Boston, MA) and washed with distilled water. The gel was dried onto paper, exposed to XAR-5 film (Kodak, Rochester, NY) at −76°C for several days and developed.

Immunodot assay. The ability of anti-pilus antibodies to bind to native antigens in the outer membrane preparations was assessed using an immunodot assay modified from the Western blot technique of Tsang *et al.*[24] The antigens examined included the outer membrane preparations from strains M43(p+) and M42(p−) and, in some experiments, outer membrane antigens that had been boiled with SDS and β-mercaptomethanol as described above for SDS–PAGE. Five μl of antigen (containing 100 μg protein/ml) were placed on 0.2 μm pore size nitrocellulose membranes (Schleicher and Schuell, Keene, NH), and allowed to dry at 37°C for 1 h, and then stored at 4°C. Immediately before use, the membranes were incubated in 0.1 M phosphate buffered saline, pH 7.3 containing 0.3% (v/v) Tween 20 (PBS-T) for 1 h at room temperature on a rotating platform. After removal of PBS-T the membranes were similarly incubated in the test rabbit serum (diluted 1 : 2000 in PBS-T). After washing, the membranes were then incubated in goat anti-rabbit IgG conjugated to horseradish peroxidase (Cappel Laboratories, Malvern, PA) diluted 1 : 2000 in PBS-T for 1 h at room temperature. The membranes were then washed and incubated in 0.5 mg/ml 3,3′ diaminobenzidine (Sigma, St. Louis, MO) in PBS-T with 0.01% (v/v) 30% H_2O_2. When complete, the reaction was stopped by washing the membranes in distilled water.

Western blot analysis. The ability of the anti-pilus antibodies to bind to immunoreactive substances resolved by SDS–PAGE was determined by the Western blot technique of Burnett, as modified by Murphy *et al.*[25] The resolved components from the SDS–PAGE gel were electrophoretically transferred onto a nitrocellulose membrane using a Trans-Blot® cell (Bio-Rad, Rachmond, CA) following the protocol supplied by the manufacturer. The membrane was dried for 1 h at 37°C, washed in PBS-T, and developed with rabbit antisera as described in the protocol for the immunodot assay. To monitor the efficiency of the transfer a strip of membrane containing a portion of the transferred antigens was stained for protein with amido black.[26]

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