Antigenic Characterization of the P6 Protein of Nontypable
*Haemophilus influenzae*

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The purpose of this study was to characterize the degree of antigenic heterogeneity or conservation of a 16,600-dalton outer membrane protein (P6) among strains of nontypable *Haemophilus influenzae*. Immunization of rabbits with P6 isolated from individual strains resulted in antibody to P6 of all 25 strains tested. The titers of antibody in the sera were similar among the strains. Whole organisms of two strains were used to immunize rabbits, and antibodies were produced to P6 of all strains tested. Monoclonal antibodies developed to P6 from mice immunized with whole cells of three different strains recognized determinants on P6 of all 25 strains tested. Finally, pooled normal human serum contained antibodies to P6 of all 25 strains assayed. These studies indicate that P6 is a highly conserved antigen on the outer membrane of nontypable *H. influenzae*.

In the past several years nontypable *Haemophilus influenzae* has become well established as an important pathogen in both adults and children (3, 14, 16–18; T. F. Murphy and M. A. Apicella, Rev. Infect. Dis., in press). The most common clinical manifestations of infections in adults are pneumonia and acute febrile tracheobronchitis, particularly in the elderly and in chronic bronchitics (3, 14, 18). The organism also causes bacteremia (18), meningitis (16, 18), postpartum sepsis (17), and sinusitis (5) in adults. In addition, nontypable *H. influenzae* causes neonatal sepsis (17) and is a frequent etiologic agent in acute otitis media in infants and children (4, 7).

The recognition of nontypable *H. influenzae* as an important human pathogen has stimulated interest in studying pathogenesis and the human immune response to infection. A 16,600-dalton outer membrane protein (OMP) referred to as P6 has been identified in all strains of *H. influenzae* studied thus far (2, 8, 12); the protein contains an epitope which is present in all strains of *H. influenzae*, both typable and nontypable (13). Antibody to P6 is present in human serum (13). More recently, we have demonstrated that P6 is a target for human bactericidal antibodies (11a). The identification of P6 as a target of human bactericidal antibodies indicates that this protein is an important surface antigen with regard to human immunity to infection and has potentially important implications in vaccine development.

To further evaluate the role of P6 in the immune response to infection, it is important to characterize the antigenic heterogeneity or antigenic conservation of determinants on the protein. For example, will immunizing with P6 from one strain of *H. influenzae* produce strain-specific antibodies, serotype-specific antibodies, or antibodies to P6 of all strains of *H. influenzae*? Previous work with monoclonal antibody 7F3 has established that P6 contains an epitope which is common to all strains of *H. influenzae* (13). However, analysis with a single monoclonal antibody allows one to draw conclusions only regarding the single determinant recognized by that antibody and not about the antigenic characteristics of the protein as a whole. The purpose of the present study is to characterize the degree of antigenic heterogeneity or antigenic conservation of P6 among strains of nontypable *H. influenzae*.

**MATERIALS AND METHODS**

**Bacterial strains.** A total of 25 isolates of nontypable *H. influenzae* was used in this study. These are listed in Table 1 along with the city in which they were isolated, the clinical sources of the isolates, and the age group of the patients from whom the strains were isolated. Isolates were kindly provided by Marilyn Loeb, Rochester, N.Y.; Daniel Mushker and Richard Wallace, Houston, Tex.; Steven Barenkamp and Dan Granoff, St. Louis, Mo.; Arnold Smith, Seattle, Wash.; and Eric Hansen, Dallas, Tex. The identity of all strains of *H. influenzae* was confirmed by growth requirement for hemin and NAD. Capsular serotypes were determined by counterimmunoelectrophoresis with reference strains and antiserum obtained from the Centers for Disease Control, Atlanta, Ga. (12, 15). Isolates were stored in Mueller-Hinton broth plus 10% glycerol at −70°C.

The OMP subtypes of the six strains used to immunize rabbits and mice were determined by our previously described method (12). These included strains 1479 (subtype 1), 3524 (subtype 2), 7502 (subtype 3), 1808 (subtype 5), 7891 (subtype 6), 5657 (subtype 7), and 39 (subtype 8).

**Isolation of P6.** A modification of the method of Munson and Granoff (9) was used to isolate P6. Outer membrane complex, which consists predominantly of OMPS and lipooligosaccharide, was first prepared (6, 11). The relative insolubility of P6 in 1% sodium dodecyl sulfate (SDS)−0.1 M Tris−0.5 M NaCl−0.1% β-mercaptoethanol, pH 8.0 (buffer B), was used to separate it from lipooligosaccharide and other OMPS.

Bacteria were grown on chocolate agar overnight at 37°C under 5% CO2. The bacteria were scraped from the plates and suspended in EDTA buffer (0.05 M Na2PO4, 0.15 M NaCl, 0.01 M EDTA, pH 7.4). This suspension was incubated at 56°C for 30 min. Cells were then disrupted by sonication on ice, with five 15-s periods of sonication at 100 W. Unbroken cells and debris were removed by centrifugation at 10,000 × g for 20 min at 4°C. The supernatant was saved, and the resulting pellet was suspended in EDTA buffer and sonicated as described above. Unbroken cells and debris were again removed by centrifugation at 10,000 × g.
Western blot assay. Gels were placed with a nitrocellulose sheet that had been previously boiled in distilled water and immersed in 0.3 M sodium citrate plus 3 M NaCl. Electrophoretic transfer was carried out in a Transphor electrophoresis unit (Hoefer Scientific Instruments, San Francisco, Calif.) at 50 V for 90 min. The electrode buffer was 0.025 M Tris (pH 8.3), 0.192 M glycine, and 20% methanol. After transfer, the nitrocellulose sheet was placed in 0.1% Tween 20 in phosphate-buffered saline (PBS-Tween) for 30 min. After the sheet was rinsed with PBS-Tween, it was incubated in appropriate dilutions of serum overnight at room temperature. The sheet was rinsed with PBS-Tween and placed in a 1:3,000 dilution of peroxidase conjugate and shaken for 1 h at room temperature. In some experiments buffer A (0.01 M Tris, 0.15 M NaCl, pH 7.4) was substituted for PBS-Tween. The sheet was rinsed and immersed in horseradish peroxidase (HRP) color development solution (0.15% H₂O₂; Bio-Rad Laboratories, Richmond, Calif.) for 45 min.

Development of monoclonal antisera. New Zealand White rabbits were immunized with solubilized P6 preparations of four strains (1479, 3524, 39, and 1808). Each animal was immunized with P6 from a single strain. The P6 preparations were emulsified with incomplete Freund adjuvant; 50 µg was injected subcutaneously on day 0 and 100 µg was injected subcutaneously on day 16. The rabbits were phlebotomized on day 25. On day 30 the rabbits received 25 µg of solubilized P6 intravenously. They were exsanguinated under anesthesia on day 34, and serum was stored at −20°C. These are referred to as anti-P6 sera.

Rabbits were immunized with whole cells of two strains (7502 and 7891). Bacteria were grown overnight on chocolate agar and suspended in sterile PBS to an optical density at 660 nm of 0.2. Rabbits were given 0.5 ml intravenously on day 0 and 0.1 ml intravenously on day 28. They were bled on day 45, and serum was stored at −20°C. These are referred to as anti-whole-cell sera.

Development of monoclonal antibodies. BALB/c mice were individually immunized intraperitoneally with whole cells of strains 3524, 1479, and 5657 on days 0 and 28. On day 32 after the initial immunization, splenic lymphocytes were fused to plasmacytoma cells by previously described methods (1, 13). Splenocytes from mice immunized with 3524 and 1479 were fused to NS1 (variant of the immunoglobulin G1 BA/c plasmacytoma P3XAg8) plasmacytoma cells. Splenocytes from the mice immunized with 5657 were fused with SP2/0 Ag14 plasmacytoma cells. Selected clones were propagated by transfer to tissue culture flasks. Larger quantities of antibodies were produced in tissue culture and by intraperitoneal injection of hybridoma cells into Pristane-primed BALB/c mice. The resulting ascitic fluid was harvested in 3 to 4 weeks and tested for specificity.

Semi quantitative dot assay. To determine the titer of anti-P6 sera to a variety of strains, whole-cell sonicates of 25 strains of nontypable H. influenzae were prepared. Several bacterial colonies from a chocolate agar plate were inoculated into 5 ml of brain heart infusion broth supplemented with hemin and NAD and incubated overnight at 37°C under 5% CO₂. The cells were centrifuged and washed in 0.01 M HEPES (N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid), pH 7.4. The pellets were suspended in 1 ml of 0.01 M HEPES buffer, pH 7.4, and sonicated for 35 s on ice. A 5-µl volume of the resulting suspension was placed on nitrocellulose.

The whole-cell sonicates on nitrocellulose were incubated overnight with serial twofold dilutions of anti-P6 rabbit
antiseras in buffer A beginning with the lowest dilution which detected only P6, as determined previously by Western blot assay. This was followed by protein A-peroxidase and substrate as described above. Preimmunization rabbit serum was incubated at the same dilution simultaneously with the homologous strain. The serum titer to P6 was defined as the highest dilution of antiserum that produced color which was darker than that produced by the preimmunization serum as detected by visual inspection.

RESULTS

Characterization of P6 preparation. P6 was isolated from strains 1479, 3524, 39, and 1808 of nontypable H. influenzae. Figure 1 is an SDS gel stained with Coomassie blue showing P6 isolated from strain 3524 along with a whole-cell preparation from the same strain. When some of the P6 preparations were initially tested, a higher-molecular-weight band (36,000 to 41,000) was detected. This corresponded to the major OMP (P2 or b/c) (8, 10), and its presence indicated incomplete solubilization of the OMP in buffer B. In these circumstances, the P6 was suspended in buffer B, incubated, and centrifuged as described above one or two more times. This resulted in disappearance of the high-molecular-weight band as detected by Coomassie blue stain of SDS gels. It is possible that P6 is contaminated with a small amount of other OMPs. However, these proteins are not detected by Coomassie blue stain, indicating that, if present, these OMPs are present in very small quantities. Silver staining of P6 preparations demonstrated that they contain no detectable lipooligosaccharide.

Polyclonal antisera to isolated P6 preparations. Antisera were tested in various dilutions in Western blot assay against whole-cell preparations of their homologous strains (1808, 1479, 3524, and 39). When the antisera were tested at lower dilutions, several bands were visible, with the P6 band being the most prominent. At higher dilutions of anti-1479 P6 serum and anti-1808 P6 serum, the antisera recognized P6 as essentially a single band. Figure 2, lane C, shows anti-1808 P6 serum at a dilution of 1:2,000 tested with a whole-cell preparation of 1808. Lane B is preimmunization serum from the same rabbit at the same dilution. Lanes D and E of Fig. 2 show pre- and post-immunization sera at dilutions of 1:1,000 from the rabbit immunized with 1479 P6 tested against a whole-cell preparation of the homologous strain. Anti-1479 P6 and anti-1808 P6 sera were relatively specific for P6 at dilutions of 1:1,000 and 1:2,000, respectively.

When tested in Western blot assay, rabbit antisera raised to P6 preparations of 3524 and 39 demonstrated antibody to P6 as well as to other bands. At greater dilutions of antisera, the higher-molecular-weight bands remained as prominent as the P6 band. Therefore, although the rabbits produced antibody to P6, the two antisera were not specific for P6.

To determine if antibody raised to P6 of one strain recognized determinants on P6 of other strains, Western blot assays were performed with anti-P6 sera. Figure 3 is a Western blot assay in which whole-cell preparations of eight strains of nontypable H. influenzae representing prototype strains of the eight OMP subtypes (12) were tested with anti-1808 P6 serum at a dilution of 1:1,000. It is demonstrated that anti-1808 P6 serum contained antibodies which recognized P6 of all eight strains. Similarly, antisera raised to 1479 P6 also contained antibodies to P6 of every strain. Higher-molecular-weight bands are present with anti-P6 sera in Fig. 3 because the antisera was used at lower dilutions than in Fig. 2, which shows a single P6 band. Anti-1808 P6 and anti-1479 P6 sera contained antibody to P6 of all 25 strains listed in Table 1 in Western blot assay. The P6 bands were similar in intensity in the strains when assayed with these antisera. To evaluate this in a more quantitative...
manner, we determined the titer of the specific anti-P6 sera in a dot assay against 25 strains.

Since these antisera were relatively specific for P6 at dilutions of >1:1,000, a positive dot assay with higher dilutions, using whole-cell sonicates, represents antibody to P6. Serial twofold dilutions of anti-1808 P6 and anti-1479 P6 sera were used to assay whole-cell sonicates of the 25 strains. Both antisera produced a positive dot assay with all 25 strains to dilutions of 1:4,000. Neither yielded a positive result at higher dilutions with the homologous strains compared with the other strains.

Polyclonal antisera to whole organisms. Figure 4 is a Western blot assay in which a 1:250 dilution of antiserum to whole organisms of strain 7502 was tested against whole-cell preparations of 16 strains of nontypable H. influenzae. Antibody to P6 of all 16 strains was present in the antiserum. Similar results were obtained with antiserum raised to whole organisms of strain 7891. It is worthy of note that the P6 band is among the most prominent bands in most lanes.

Monoclonal antibodies to P6. Three monoclonal antibodies to P6 were developed from mice which were immunized with whole cells of three strains of nontypable H. influenzae. Antibody 7F3 was from mice immunized with 3524 and has been characterized in detail previously (13). Antibody 4G4 was developed to 1479 and antibody 4F7 was developed to 5657. All three monoclonal antibodies are immunoglobulin G, and all three antibodies recognize P6 as a single band in Western blot assays of whole-cell preparations. Dot assays were performed with the antibodies to whole-cell preparations of the 25 strains of nontypable H. influenzae. The determinants recognized by each of the three monoclonal antibodies were present in all 25 strains by dot assay.

Normal human serum. To determine whether humans produce antibodies to P6 of various strains, pooled normal human serum obtained from six healthy adults was tested in Western blot assay to whole-cell preparations from 25 strains. Figure 5 is a Western blot assay which shows that human serum contains antibodies to P6 of seven strains of nontypable H. influenzae representing the OMP subtypes (12). Normal human serum contains antibodies to P6 of all 25 strains listed in Table 1 by Western blot assay.

DISCUSSION

To summarize the results of the present study, immunization of rabbits with P6 isolated from individual strains of nontypable H. influenzae resulted in antibody to P6 of all 25 strains tested. Second, the titer of antibody to P6 in these antisera to the homologous strains was similar in titer to P6 of multiple strains. Third, when whole organisms of two strains of nontypable H. influenzae were used to immunize rabbits, antibodies were produced to P6 of multiple strains. Fourth, monoclonal antibodies developed from individual mice immunized with whole cells from three different strains recognized determinants on P6 of all 25 strains tested. Finally, pooled normal human serum contained antibodies to P6 of all 25 strains assayed.

It should be noted that the strains used in this study were isolated from a broad range of patients in diverse clinical settings from several cities in the United States. Prototype strains of all eight OMP subtypes (12) and all six OMP serotypes (11) were included among the 25 strains. The antisera used to study the strains were raised in different species by immunization with different preparations, and all showed similar results in that antibodies were present to P6 of all 25 strains tested in all antisera. These included monoclonal rabbit antisera to both isolated P6 preparations and whole organisms, mouse monoclonal antibodies raised to

FIG. 3. Western blot assay in which all lanes were assayed with anti-1808 P6 serum at dilutions of 1:1,000. All lanes contained whole-cell preparations. Lanes: a, 3198; b, 5657; c, 7891; d, 4971; e, C7961; f, 7502; g, 2019; h, 1479. Serum was followed by protein A-peroxidase and Bio-Rad HRP color developer. Molecular weights are noted in thousands.

FIG. 4. Western blot assays in which all lanes were assayed with anti-7502 whole-cell serum at a dilution of 1:250. All lanes contained whole-cell preparations. Lanes: a, 616; b, 601; c, SA100; d, 4971; e, \( C7961; f, 7502; g, 2019; h, 1479; j, 1808; k, 8097; l, 7891; m, 5657; n, \( S173; o, C378; p, C1327; \) p, C973. Serum was followed by protein A-peroxidase and Bio-Rad HRP color developer. Molecular weights are noted in thousands.
whole organisms, and human serum from healthy adults. Taken together, these data indicate that P6 is a highly conserved antigen on the outer membrane of nontypable H. influenzae. The protein might be composed of multiple antigenic determinants which are common to many strains of nontypable H. influenzae. Alternatively, P6 might contain an immunodominant determinant present on P6 of all strains.

Rabbit antisera to isolated P6 of strains 3524 and 39 contained antibodies to several proteins including P6. The most likely reason was that the preparations used to immunize the rabbits contained small amounts of contaminating proteins. Therefore, these antisera were not used to quantitate antibody to P6. Since antisera to P6 of 1808 and 1479 were relatively specific for P6 at dilutions of >1:1,000, dot assays that used whole-cell sonicates were used to quantitate antibody to P6 of the 25 strains. Although these antisera were specific for P6 at high dilutions, it should be pointed out that the whole-cell sonicates contained mixtures of antigens. Therefore, this assay should be considered semiquantitative. With this reservation, the antisera contained similar antibody titers to P6 of all strains by serial twofold dilutions. The homologous strain was indistinguishable in antibody titer from the others in this semiquantitative assay. The results indicate that the isolated P6 preparations did not induce strain-specific or serotype-specific antibodies, but rather P6 stimulated antibodies to determinants which are common to many strains of nontypable H. influenzae.

The monoclonal antibodies used in this study were developed to whole cells of three strains of different subtypes. Each of the three antibodies recognized a determinant on all 25 strains tested. It is yet to be determined whether these antibodies recognize the same or different determinants.

Preliminary studies with these antisera to test type b strains indicate that extensive antigenic cross-reactivity exists between P6 of nontypable and type b strains. This has been demonstrated with both polyclonal and monoclonal antibodies. Indeed, previous work has shown that 73 of 73 type b strains contain the epitope recognized by monoclonal antibody 7F3 (13). A systematic study will be important to elucidate the antigenic characteristics of P6 in type b strains.

The antigenic characteristics of P6 suggest that this protein might be useful as a vaccine component to prevent infections caused by H. influenzae. P6 is a target for human bactericidal antibodies, indicating that the protein might induce protective antibodies (11a). Antibodies to P6-cell wall complex prevent meningitis in an infant rat model (9). In addition, the results of the current study indicate that P6 is antigenically conserved among strains of nontypable H. influenzae, indicating that antibodies generated by P6 of one strain recognize P6 of many or all strains of the bacterium. It will be critical to determine whether antibodies to P6 are indeed protective and, if so, whether the determinants on P6 to which protective antibodies are directed are conserved among strains. Once these determinants are identified, it would be feasible to develop a protein vaccine to prevent infections due to nontypable H. influenzae. In addition, a conjugate vaccine which includes P6 might be effective in preventing infections caused by strains of H. influenzae type b.

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LITERATURE CITED


