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Molecular Conservation of the P6 Outer Membrane Protein among Strains of *Haemophilus influenzae*: Analysis of Antigenic Determinants, Gene Sequences, and Restriction Fragment Length Polymorphisms

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Infections caused by *Haemophilus influenzae* are a major worldwide health problem. In particular, nontypeable strains of *H. influenzae* are a common cause of otitis media in infants and children. A vaccine to prevent these infections would result in the prevention of substantial morbidity and cost savings. A problem in identifying an appropriate vaccine antigen has been the enormous antigenic heterogeneity among nontypeable strains of *H. influenzae*. The present study was undertaken to characterize the conservation of the P6 outer membrane protein (~16,000 daltons) among strains of *H. influenzae*. A total of 20 type b strains and 20 nontypeable strains of diverse geographic and clinical origins was studied. Three approaches were taken. (i) Antigenic determinants recognized by monoclonal and polyclonal antibodies were present on P6 in all 40 strains tested. The molecular weight of P6 was identical in all strains. (ii) Comparison of the DNA sequences of the P6 genes from three epidemiologically and serologically unrelated strains demonstrated 100% homology at the amino acid level and 97 to 99% homology at the nucleotide level. (iii) Restriction fragment length polymorphism analysis demonstrated that the P6 gene and flanking sequences were highly conserved among all strains. These three independent series of experiments indicated that the P6 protein is highly conserved among strains of *H. influenzae*. P6 should receive serious consideration for inclusion in a vaccine to prevent infections caused by nontypeable *H. influenzae*.

Haemophilus influenzae is an important human pathogen in children and adults. *H. influenzae* type b is the most common cause of meningitis in infants and children in the United States (9, 42). These encapsulated strains also cause other invasive diseases in infants and children, including epiglottitis, bacteremia, pneumonia, cellulitis, and others (4, 9, 14, 20, 42). Nontypeable or nonencapsulated strains affect different patient populations and cause predominantly noninvasive mucosal infections (24). These strains are the second most common cause of otitis media in infants and children after pneumococci (6, 7, 18, 40). Nontypeable *H. influenzae* is also an important cause of respiratory tract infections in adults, particularly the elderly and patients with chronic bronchitis, and in children in the developing world (5, 30, 39, 41, 43, 45).

In view of the morbidity and mortality associated with *H. influenzae* infections, efforts have focused on the development of vaccines to prevent these infections. A polysaccharide vaccine consisting of the type b capsule is effective in children over the age of 2 years (35, 36); however, the majority of infections caused by type b strains occur in infants under 2 years old (9, 42). Several conjugate vaccines in which the capsular polysaccharide is conjugated to carrier proteins have been developed, and these are effective in generating antibody to the capsule in most infants (1–3, 11, 19, 44, 46). A field trial in Finland demonstrated that one of the conjugate vaccines was effective in infants (12). Ongoing efficacy trials will determine the potential role of these vaccines in other populations.

The approach to developing a vaccine to prevent infections by nontypeable strains of *H. influenzae* must be different because these strains do not express a polysaccharide capsule (16). A growing body of evidence indicates that the P6 protein is a potential vaccine antigen for nontypeable *H. influenzae*. P6 is a 16,000-dalton outer membrane protein (OMP) which constitutes 1 to 5% of the protein content of the outer membrane (22). The protein expresses epitopes on the surface of the intact bacterium, and these epitopes are accessible to mouse monoclonal antibodies (29), rabbit polyclonal antibodies (22), and human serum antibodies (26). Three lines of evidence suggest that antibodies to P6 are protective. First, antibodies to P6 are protective in the infant rat model of *H. influenzae* type b infection (22). Second, P6 of nontypeable *H. influenzae* is a target for human bactericidal antibodies (26). Third, antiserum raised to purified P6 is bactericidal for all strains of *H. influenzae* tested (15).

Since antibodies to P6 are potentially protective and since P6 may be incorporated into vaccines, it is important to establish the degree of antigenic heterogeneity of P6 among strains. We previously demonstrated that antibodies to P6 of individual strains recognized determinants on P6 of all strains of nontypeable *H. influenzae* tested (25). These data were consistent with two hypotheses. (i) The entire P6 protein is conserved among strains. (ii) P6 expresses a limited number of immunodominant epitopes, and immuni-

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zation results in the production of antibodies predominantly to these epitopes.

In view of the importance of the P6 protein as a vaccine antigen, the present study was designed to analyze the antigenic and molecular conservation of the P6 protein among strains of nontypeable and type b *H. influenzae*. This analysis involved three approaches: (i) studies with monoclonal and polyclonal antibodies to P6, (ii) determination and comparison of P6 gene sequences, and (iii) analysis of restriction fragment length polymorphisms (RFLPs) in and around the P6 gene in 40 strains.

TABLE 1. Five P6 oligonucleotide probes used in RFLP studies

Oligonucleotide probe	Nucleotide position in pBUD5[a]	Strand	Sequence
18G	138	Gene	AACAACGATGCTGCAGG
P	212	Gene	AATACCGTTTATTTCGG
19syn2	244	Opposite	GTATTCACCGGTGATGT
18syn2	410	Gene	GGTAAAGGTGTTGATGC
19A	507	Opposite	ACGCTAACACTGCACGA

[a] Plasmid pBUD5 contains the P6 gene of strain 1479 (34).

MATERIALS AND METHODS

Bacteria. A total of 20 strains of *H. influenzae* type b and 20 strains of nontypeable *H. influenzae* was used. Strains were chosen so that a diverse collection of isolates could be studied with regard to molecular conservation of the P6 gene and protein among strains. The type b strains included 19 different electrophoretic types and therefore represent various evolutionary distances (31–33, 37, 38). The nontypeable strains represent a variety of OMP subtypes (27, 28), OMP serotypes (23), and lipooligosaccharide serotypes (8) and were recovered from several different cities in the United States.

Antibodies. Monoclonal antibodies 7F3 and 4G4 recognize closely related but different epitopes on the P6 protein and have been described previously (25, 29). Antibody 7F3 was raised to nontypeable *H. influenzae* 3524, and antibody 4G4 was raised to *H. influenzae* 1479. Both antibodies recognize surface-exposed epitopes on P6 and bind P6 in several immunoassays, including immunoblot assay, immunofluorescence, immunoelectron microscopy, and enzyme-linked immunosorbent assay.

Anti-1808 P6 and anti-1479 P6 are individual rabbit polyclonal antisera raised to purified P6 of strains 1808 and 1479 (25). P6 was purified as previously described and emulsified with incomplete Freund's adjuvant; 50 μg was injected subcutaneously on day 0, and 100 μg was injected subcutaneously on day 16. On day 30, each rabbit received 25 μg of solubilized P6 intravenously. Serum was collected on day 34 after the initial immunization.

SDS-PAGE and immunoblot assays. Whole-cell lysates of bacterial strains were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 15% separating gels as previously described (27). For preparation of whole-cell lysates, bacteria were grown on chocolate agar overnight. Cells were harvested, suspended in 0.01 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (pH 7.4), and centrifuged. The resulting bacterial cell pellet was suspended in 1 ml of HEPES and sonicated for 35 s to disrupt cells. Antigen preparations were heated at 100°C for 5 min in a sample buffer containing 0.06 M Tris (pH 6.8), 1.2% SDS, 5% β-mercaptoethanol, 11.9% glycerol, and 0.003% bromophenol blue. Immunoblot assays were performed by electrophoretic transfer to nitrocellulose. P6 was detected with monoclonal and polyclonal antibodies by previously described methods (25).

Molecular cloning and sequencing. To clone the P6 gene from strain MinnA, we used monoclonal antibody 7F3 to immunoscreen a lambda EMBL3 genomic library prepared from DNA of strain MinnA. This library has been described previously (21). Positive plaques were picked and replated until all plaques from a clone tested positive in a plaque immunoassay. DNA from a positive clone was digested with *Bgl*II and *Bam*HI, and the resulting 737-bp fragment containing the P6 gene was excised from the gel and ligated into the *Bam*HI site of M13mp18. The insert from this clone was excised with appropriate restriction enzymes and ligated into M13mp19 to allow sequencing of the other strand. The sequences of both strands of one clone from the EMBL3 library were independently determined. Dideoxy sequencing was performed with Sequenase (U.S. Biochemicals) in accordance with the manufacturer's instructions.

Computer-assisted analysis of the protein and nucleotide sequences was performed with the Genetics Computer Group Sequence Analysis Software Package.

RFLPs. (i) Purification of DNA. Chromosomal DNA was isolated from the 40 strains of *H. influenzae* as follows. The bacterial pellet from a 100-ml overnight culture was suspended in 8 ml of TE buffer (0.01 M Tris, 0.001 M EDTA [pH 7.6]). EDTA was added to a final concentration of 0.005 M, and SDS was added to 0.5%. The mixture was heated at 60°C for 30 min. Proteinase K was added to 100 μg/ml, and the suspension was incubated at 65°C for 60 to 90 min. The nucleic acids were extracted twice with phenol-chloroform and twice with chloroform alone. The nucleic acids were precipitated by the addition of a 10% volume of 3 M sodium acetate and cold ethanol to 80% of the volume, and the mixture was centrifuged. The pellet was suspended in 2 ml of 0.05 M Tris (pH 8), and RNA was digested by the addition of RNase A to a final concentration of 20 μg/ml. The DNA was extracted and precipitated as described above and dissolved in 2 ml of TE buffer. Each DNA preparation was assayed by measuring the ratio of the optical density at 260 nm to the optical density at 280 nm and by agarose gel electrophoresis.

(ii) Digestion of DNA with restriction enzymes. Genomic DNA was digested with *Bgl*II and *Bam*HI together with buffers recommended by the manufacturers. When DNA was digested with *Bgl*II, *Bam*HI, and *Pvu*II, the sample was first incubated with *Pvu*II and then the DNA was extracted and ethanol precipitated. The DNA was then digested with the other two enzymes together.

(iii) Agarose gel electrophoresis and Southern blotting. DNA digested with *Bgl*II and *Bam*HI was subjected to electrophoresis on both 0.7% agarose gels and 3% NuSieve gels containing 0.5% agarose. Samples containing smaller fragments were resolved on gels composed of NuSieve plus 0.5% agarose. Southern blotting was performed with Nytran (Schleicher & Schuell, Inc., Keene, N.H.) in accordance with the manufacturer's recommendations.

Oligonucleotides were synthesized at the University at Buffalo Microsequencing Facility. The sequences of the five oligonucleotides are noted in Table 1. For hybridization in Southern blot assays, oligonucleotides were end labeled with [^{32}P]ATP by use of T4 polynucleotide kinase. Hybridizations and washes were performed at 5 to 7°C below the temperature of dissociation for the probes. This was 45 to 47°C for all

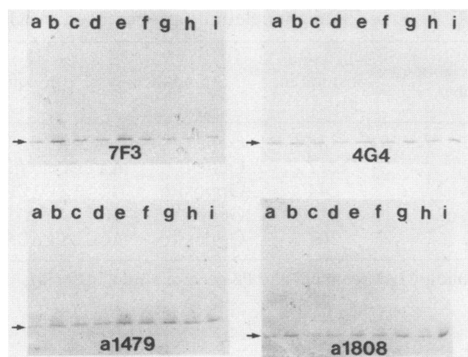

FIG. 1. Immunoblot assays of whole-cell lysates of the following nine strains of *H. influenzae* type b (lanes): a, 1052; b, 1053; c, 1054; d, 1059; e, 1060; f, 1061; g, 1062; h, 1063; and i, 1064. Blots on top were assayed with monoclonal antibodies 7F3 and 4G4. Blots on bottom were assayed with polyclonal antisera anti-1479 P6 and anti-1808 P6. The arrows note the P6 band.

five probes. Autoradiography was performed overnight at −70°C.

RESULTS

Antigenic conservation. Whole-cell preparations from each of the 20 type b strains and 20 nontypeable strains were subjected to SDS-PAGE, transferred to nitrocellulose, and assayed separately with four antisera to P6. These included monoclonal antibodies 7F3 and 4G4 and the two polyclonal antisera, anti-1808 P6 and anti-1479 P6. All four antisera recognized a 16,000-dalton band in all 40 strains. Figure 1 shows nine type b strains assayed with each of the four antisera. The P6 band in each of the 40 strains showed an identical migration pattern in SDS-PAGE and the immunoblot assay. These data indicate that the determinants recognized by these four antisera are conserved among strains of *H. influenzae*. They further indicate that the molecular mass of P6 is identical among strains within the experimental accuracy of SDS-PAGE.

Sequence analysis. An EMBL3 library of genomic DNA from strain MinnA was screened with antibody 7F3. DNA from a clone that was reactive with 7F3 was digested with *Bgl*II and *Bam*HI. The resulting 738-bp fragment containing the P6 gene was excised from the gel and ligated into the *Bam*HI site of M13mp18, and the nucleotide sequence was determined. The sequence of the P6 gene was compared with the previously reported sequences of the P6 genes from the type b strain Eagan (10) and the nontypeable strain 1479 (34). The sequences of the P6 genes from strains MinnA and Eagan differed in two nucleotides. In the MinnA gene, an A

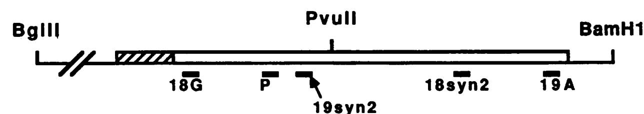

FIG. 3. Schematic diagram of the P6 gene and flanking regions. The approximate locations of the five oligonucleotide probes are shown below the P6 gene. The hatched area represents the leader peptide. The *Bgl*II-*Bam*HI fragment is 738 bp, the *Bgl*II-*Pvu*II fragment is 462 bp, and the *Pvu*II-*Bam*HI fragment is 274 bp.

was present at nucleotide 342 (translational start site defined as position 1) and a C was present at position 414. The P6 sequence of the strain MinnA gene differed by 13 nucleotides from the P6 sequence of the strain 1479 gene. Six of these changes were localized to a 39-bp region of the P6 gene (Fig. 2). The remaining seven nucleotide differences were located in the first two-thirds of the gene but were not clustered in any particular region. The genes of all three strains translated to an identical 153-amino-acid sequence including a leader peptide of 19 amino acids. Therefore, comparison of the P6 genes indicates 97% homology at the nucleotide level and 100% homology at the amino acid level.

RFLPs. As noted above, the P6 gene from strain MinnA was subcloned as a 738-bp *Bam*HI-*Bgl*II fragment. The positions of these sites as well as an internal *Pvu*II site are shown in Fig. 3. To determine whether the *Bgl*II and *Bam*HI sites surrounding the P6 gene were conserved among both type b and nontypeable *H. influenzae* strains, we prepared genomic DNA from 40 strains and characterized it by Southern hybridization.

Genomic DNA was digested with *Bgl*II and *Bam*HI and subjected to agarose gel electrophoresis. After transfer to a nylon membrane, the resulting Southern blot was incubated with an oligonucleotide probe, 18syn2 (Fig. 3 and Table 1). The probe hybridized to a single band of approximately 738 bp in each of the 40 strains (Fig. 4).

To further characterize the conservation of the P6 gene, we digested genomic DNA with *Pvu*II and then with *Bgl*II and *Bam*HI. A single *Pvu*II site is located in the P6 gene and yields fragments of 463 and 274 bp when DNA of strains MinnA and 1479 is digested with these three enzymes, respectively. To detect RFLPs, we synthesized four oligonucleotide probes that correspond to sequences in different regions of the P6 gene of strain 1479. These are depicted diagrammatically in Fig. 3 and described further in Table 1. A fifth oligonucleotide probe, probe P, was synthesized with sequence data from the type b strains and corresponded to a region of the P6 gene with some differences in sequence between strains 1479 and MinnA. Probes 19A and 18syn2 were used to detect the 274-bp fragment, and probes 18G, P, and 19syn2 were used individually to detect the 463-bp fragment.

Minn A	AAT	ACC	GTT	TAT	TTC	GGT	TTT	GAT	AAA	TAT	GAC	ATT	ACT	GGT	GAA	TAC
	Asn	Thr	Val	Tyr	Phe	Gly	Phe	Asp	Lys	Tyr	Asp	Ile	Thr	Gly	Glu	Tyr
1479	C		A		T					C		C	C			
	AAT	ACC	GTT	TAT	TTC	GG					AC	ATC	ACC	GGT	GAA	TAC
	PROBE P										**PROBE 19 syn 2**					

FIG. 2. Nucleotides 145 to 192 of the P6 gene sequence of strain MinnA. Nucleotide differences in this region of the P6 gene from strain 1479 are shown below the translated strain MinnA P6 gene sequence. Six silent nucleotide changes are observed in this portion of the gene. Oligonucleotides P and 19syn2 were generated and used to determine the conservation of this portion of the P6 gene in other *H. influenzae* strains. Probe 19syn2 actually represents the opposite strand but is depicted here as the gene strand.

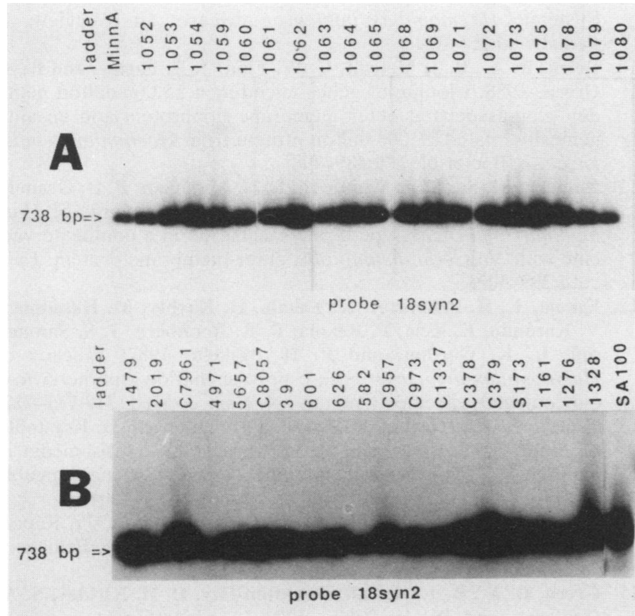

FIG. 4. Autoradiographs of Southern blot assays of genomic DNA from 40 strains of *H. influenzae* digested with *Bgl*II and *Bam*HI. The blots were assayed with oligonucleotide 18syn2. (A) Type b strains. (B) Nontypeable strains.

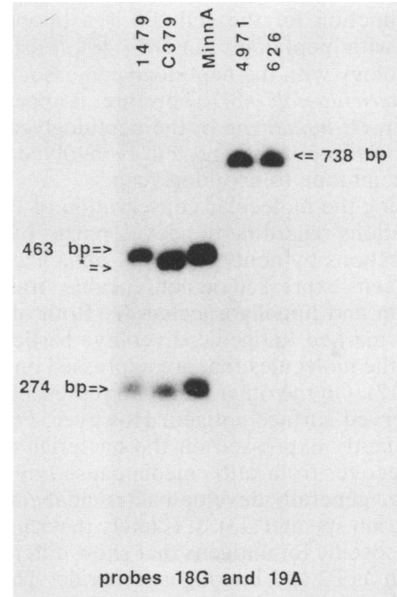

FIG. 5. Autoradiograph of a Southern blot assay. Genomic DNA from five strains was digested with *Bgl*II, *Bam*HI, and *Pvu*II. The blot was assayed with oligonucleotides 18G and 19A.

Genomic DNA from all 40 strains was digested with the three enzymes (*Bgl*II, *Bam*HI, and *Pvu*II), and the resulting fragments were probed individually with all five oligonucleotide probes. The results of the RFLP analysis and hybridization with the five probes are shown in Table 2.

Thirty-one strains showed identical patterns, and 5 additional strains differed only in that probe P showed somewhat less prominent hybridization. Since a difference of 3 of 17 nucleotides in probes P and 19syn2 determines a positive versus a negative result (e.g., 1479 and MinnA in Table 2), the partial hybridization seen in some strains is a result of a 1- or 2-bp difference. Figure 5 is an autoradiograph of a Southern blot showing RFLPs of five strains, including two strains (1479 and MinnA) with typical fragment sizes, two strains lacking the *Pvu*II site (4971 and 626), and one strain (C379) with a slightly smaller *Bgl*II-*Pvu*II fragment.

Taken together, these data indicate that the P6 gene and flanking sequences are highly conserved among strains of *H. influenzae*, including type b and nontypeable strains. We conclude that the antigenic similarities of the P6 protein are due to the conservation of the P6 gene among strains of *H. influenzae*.

TABLE 2. RFLP analysis and hybridization with oligonucleotide probes corresponding to P6 sequences of 40 strains of *H. influenzae*

No. of strains		Pattern[a] with the following probe(s) or site:			
Type b	Nontypeable	18G, 18syn2, and 19A	*Pvu*II site present	P	19syn2
16[b]	15[c]	+	Yes	+	−
3	2	+	Yes	±	−
0	2	+	No	−	+
1	0	+	Yes	±	+
0	1[d]	+	Yes	−	+

[a] +, hybridization of probe in Southern blot; ±, weak hybridization in Southern blot; −, negative result in Southern blot.
[b] Strains MinnA and Eagan have this pattern.
[c] Two of these strains (C379 and 1276) have a slightly smaller *Bgl*II-*Pvu*II fragment.
[d] Strain 1479 has this pattern.

DISCUSSION

Three independent series of experiments reported in this study indicate that the P6 protein is highly conserved among strains of *H. influenzae* at both the protein and DNA levels. (i) Antigenic determinants recognized by monoclonal and polyclonal antibodies are present on P6 in all 40 strains tested. The molecular weight of P6 is identical in all strains. (ii) Comparison of the DNA sequences of the P6 genes from three epidemiologically and serologically unrelated strains demonstrates 100% homology at the amino acid level and 97 to 99% homology at the nucleotide level. (iii) RFLP analysis demonstrates that the P6 gene and flanking sequences are highly conserved among all strains.

In this investigation, the 40 strains were chosen so that the P6 genes of strains which are genetically distant within the species were studied. Electrophoretic typing has established that type b strains are basically clonal, while nontypeable strains show substantial genetic diversity (31–33, 37, 38). The 20 type b strains represent different electrophoretic types (33); the 20 nontypeable strains represent different OMP subtypes (27, 28), OMP serotypes (23), and different lipooligosaccharide antigens (8). In particular, the P2 protein of the nontypeable strains shows enormous antigenic heterogeneity (17). Despite the diversity of the strains, each strain had a P6 gene that was highly conserved. One must conclude that there is strong selective pressure on *H. influenzae* to continue to express this highly conserved surface antigen. Therefore, it is likely that P6 performs an important function for the cell.

The function of P6 has not been determined directly. However, indirect evidence indicates that it performs a

structural function for the cell. P6 is a lipoprotein and is associated with peptidoglycan (47, 48). Furthermore, P6 shares homology with the peptidoglycan-associated lipoprotein of *Escherichia coli* (48). Therefore, it appears that P6 is the analog in *H. influenzae* of the peptidoglycan-associated lipoprotein in *E. coli*. This protein is involved in anchoring the outer membrane to peptidoglycan.

Establishing the molecular conservation of P6 has important implications regarding the development of a vaccine to prevent infections by nontypeable *H. influenzae*. The major surface antigens expressed on nontypeable strains are the P2 porin protein and lipooligosaccharide. Both of these molecules show marked antigenic diversity, particularly in the portions of the molecules that are expressed on the bacterial surface (8, 17). On the other hand, P6 represents an antigenically conserved surface antigen. However, P6 appears not to be abundantly expressed on the bacterial surface. Children who recover from otitis media caused by nontypeable *H. influenzae* generally develop bactericidal antibody that is relatively strain specific (13). It is likely that this bactericidal antibody is specific for antigens that show differences among strains, such as P2 and lipooligosaccharide. The expression of only small amounts of P6 on the bacterial surface ''hides'' this conserved antigen, thus making recurrent infections possible. Future investigations should focus on determining whether the induction of an antibody to this conserved, potentially protective, ''hidden'' antigen will protect infants and children from otitis media.

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REFERENCES

1. **Anderson, P., M. Pichichero, K. Edwards, C. R. Porch, and R. Insel.** 1987. Priming and induction of *Haemophilus influenzae* type b capsular antibodies in early infancy by Dpo20, an oligosaccharide-protein conjugate vaccine. J. Pediatr. **111:**644–650.
2. **Anderson, P., M. E. Pichichero, and R. A. Insel.** 1985. Immunization of 2-month-old infants with protein-coupled oligosaccharides derived from the capsule of *Haemophilus influenzae* type b. J. Pediatr. **107:**346–351.
3. **Anderson, P. W., M. E. Pichichero, R. A. Insel, R. Betts, R. Eby, and D. H. Smith.** 1986. Vaccines consisting of periodate-cleaved oligosaccharides from the capsule of *Haemophilus influenzae* type b coupled to a protein carrier: structural and temporal requirements for priming in the human infant. J. Immunol. **137:**1181–1186.
4. **Berenberg, W., and S. Kevy.** 1958. Acute epiglottitis in childhood: a serious emergency readily recognized at the bedside. N. Engl. J. Med. **258:**870–874.
5. **Berk, S. L., S. A. Holtsclaw, S. L. Wiener, and J. K. Smith.** 1982. Nontypeable *Haemophilus influenzae* in the elderly. Arch. Intern. Med. **142:**537–539.
6. **Bluestone, C. D.** 1982. Current concepts in otolaryngology. Otitis media in children: to treat or not to treat? N. Engl. J. Med. **306:**1399–1404.
7. **Bluestone, C. D., and J. O. Klein.** 1983. Otitis media with effusion, atelectasis and eustachian tube dysfunction, p. 356. *In* C. D. Bluestone and S. E. Stool (ed.), Pediatric otolaryngology. The W. B. Saunders Co., Philadelphia.
8. **Campagnari, A. A., M. R. Gupta, K. C. Dudas, T. F. Murphy, and M. A. Apicella.** 1987. Antigenic diversity of lipooligosaccharides of nontypeable *Haemophilus influenzae*. Infect. Immun. **55:**882–887.
9. **Dajani, A. S., B. I. Asmar, and M. C. Thirmoorthi.** 1979. Systemic *Haemophilus influenzae* disease: an overview. J. Pediatr. **94:**355–364.
10. **Deich, R. A., B. J. Metcalf, C. W. Finn, J. E. Farley, and B. A. Green.** 1988. Cloning of genes encoding a 15,000-dalton peptidoglycan-associated outer membrane lipoprotein and an antigenically related 15,000-dalton protein from *Haemophilus influenzae*. J. Bacteriol. **170:**489–498.
11. **Einhorn, M. S., G. A. Weinberg, E. L. Anderson, P. D. Granoff, and D. M. Granoff.** 1986. Immunogenicity in infants of *Haemophilus influenzae* type b polysaccharide in a conjugate vaccine with *Neisseria meningitidis* outer-membrane protein. Lancet **i:**299–302.
12. **Eskola, J., H. Peltola, A. K. Takala, H. Kayhty, M. Hakulinen, V. Karanko, E. Kela, P. Rekola, P. R. Ronnberg, J. S. Samuelson, L. K. Gordon, and P. H. Makela.** 1987. Efficacy of *Haemophilus influenzae* type b polysaccharide-diphtheria toxoid conjugate vaccine in infancy. N. Engl. J. Med. **317:**717–722.
13. **Faden, H., J. Bernstein, L. Brodsky, J. Stanievich, D. Krystofik, C. Shuff, J. J. Hong, and P. L. Ogra.** 1989. Otitis media in children. I. The systemic immune response to nontypeable *Haemophilus influenzae*. J. Infect. Dis. **160:**999–1004.
14. **Ginsburg, C. M., J. B. Howard, and J. D. Nelson.** 1979. Report of 65 cases of *Haemophilus influenzae* pneumonia. Pediatrics **64:**283–286.
15. **Green, B. A., B. J. Metcalf, T. Quinn-Dey, D. H. Kirkley, S. A. Quataert, and R. A. Deich.** 1990. A recombinant non-fatty acylated form of the Hi-PAL (P6) protein of *Haemophilus influenzae* elicits biologically active antibody against both nontypeable and type b *H. influenzae*. Infect. Immun. **58:**3272–3278.
16. **Gyorkey, F., D. Musher, P. Gyorkey, A. Goree, and R. Baughn.** 1984. Nontypeable *Haemophilus influenzae* are unencapsulated both in vivo and in vitro. J. Infect. Dis. **149:**518–522.
17. **Haase, E. M., A. A. Campagnari, J. Sarwar, M. Shero, M. Wirth, C. U. Cumming, and T. F. Murphy.** 1991. Strain-specific and immunodominant surface epitopes of the P2 porin protein of nontypeable *Haemophilus influenzae*. Infect. Immun. **59:**1278–1284.
18. **Howie, V. M., J. H. Ploussard, and J. Sloyer.** 1975. The otitis prone condition. Am. J. Dis. Child. **129:**676–678.
19. **Lenoir, A. A., P. D. Granoff, and D. M. Granoff.** 1987. Immunogenicity of *Haemophilus influenzae* type b polysaccharide-*Neisseria meningitidis* outer membrane protein conjugate vaccine in 2- to 6-month-old infants. Pediatrics **80:**283–287.
20. **Marshall, R., D. W. Teele, and J. O. Klein.** 1979. Unsuspected bacteremia due to *Haemophilus influenzae*: outcome in children not initially admitted to hospital. J. Pediatr. **95:**690–695.
21. **Munson, R., Jr., and S. Grass.** 1988. Purification, cloning, and sequence of outer membrane protein P1 of *Haemophilus influenzae* type b. Infect. Immun. **56:**2235–2242.
22. **Munson, R. S., Jr., and D. M. Granoff.** 1985. Purification and partial characterization of outer membrane proteins P5 and P6 from *Haemophilus influenzae* type b. Infect. Immun. **49:**544–549.
23. **Murphy, T. F., and M. A. Apicella.** 1985. Antigenic heterogeneity of outer membrane proteins of nontypeable *Haemophilus influenzae* is a basis for a serotyping system. Infect. Immun. **50:**15–21.
24. **Murphy, T. F., and M. A. Apicella.** 1987. Nontypeable *Haemophilus influenzae*: a review of clinical aspects, surface antigens, and the human immune response to infection. Rev. Infect. Dis. **9:**1–15.
25. **Murphy, T. F., L. C. Bartos, A. A. Campagnari, M. B. Nelson, and M. A. Apicella.** 1986. Antigenic characterization of the P6 protein of nontypeable *Haemophilus influenzae*. Infect. Immun. **54:**774–779.
26. **Murphy, T. F., L. C. Bartos, P. A. Rice, M. B. Nelson, K. C. Dudas, and M. A. Apicella.** 1986. Identification of a 16,600-dalton outer membrane protein on nontypeable *Haemophilus influenzae* as a target for human serum bactericidal antibody. J. Clin. Invest. **78:**1020–1027.
27. **Murphy, T. F., J. M. Bernstein, D. M. Dryja, A. A. Campagnari, and M. A. Apicella.** 1987. Outer membrane protein and lipooli-

gosaccharide analysis of paired nasopharyngeal and middle ear isolates in otitis media due to nontypeable *Haemophilus influenzae*: pathogenetic and epidemiological observations. J. Infect. Dis. **156**:723–731.

28. **Murphy, T. F., K. C. Dudas, J. M. Mylotte, and M. A. Apicella.** 1983. A subtyping system for nontypeable *Haemophilus influenzae* based on outer-membrane proteins. J. Infect. Dis. **147**: 838–846.
29. **Murphy, T. F., M. B. Nelson, K. C. Dudas, J. M. Mylotte, and M. A. Apicella.** 1985. Identification of a specific epitope of *Haemophilus influenzae* on a 16,600-dalton outer membrane protein. J. Infect. Dis. **152**:1300–1307.
30. **Musher, D. M., K. R. Kubitschek, J. Crennan, and R. E. Baughn.** 1983. Pneumonia and acute febrile tracheobronchitis due to *Haemophilus influenzae*. Ann. Intern. Med. **99**:444–450.
31. **Musser, J. M., S. J. Barenkamp, D. M. Granoff, and R. K. Selander.** 1986. Genetic relationships of serologically nontypeable and serotype b strains of *Haemophilus influenzae*. Infect. Immun. **52**:183–191.
32. **Musser, J. M., D. M. Granoff, P. E. Pattison, and R. K. Selander.** 1985. A population genetic framework for the study of invasive diseases caused by serotype b strains of *Haemophilus influenzae*. Proc. Natl. Acad. Sci. USA **82**:5078–5082.
33. **Musser, J. M., J. S. Kroll, E. R. Moxon, and R. K. Selander.** 1988. Evolutionary genetics of the encapsulated strains of *Haemophilus influenzae*. Proc. Natl. Acad. Sci. USA **85**:7758–7762.
34. **Nelson, M. B., M. A. Apicella, T. F. Murphy, H. Vankeulen, L. D. Spotila, and D. Rekosh.** 1988. Cloning and sequencing of *Haemophilus influenzae* outer membrane protein P6. Infect. Immun. **56**:128–134.
35. **Peltola, H., H. Kayhty, A. Sivonen, and P. H. Makela.** 1977. *Haemophilus influenzae* type b capsular polysaccharide vaccine in children: a double-blind field study of 100,000 vaccinees 3 months to 5 years of age in Finland. Pediatrics **60**:730–737.
36. **Peltola, H., H. Kayhty, M. Virtanen, and P. H. Makela.** 1984. Prevention of *Hemophilus influenzae* type b bacteremic infections with the capsular polysaccharide vaccine. N. Engl. J. Med. **310**:1561–1566.
37. **Porras, O., D. A. Caugant, B. Gray, T. Lagergard, B. R. Levin, and C. Svanborg-Eden.** 1986. Difference in structure between type b and nontypeable *Haemophilus influenzae* populations. Infect. Immun. **53**:79–89.
38. **Porras, O., D. A. Caugant, T. Lagergard, and C. Svanborg-Eden.** 1986. Application of multilocus enzyme gel electrophoresis to *Haemophilus influenzae*. Infect. Immun. **53**:71–78.
39. **Stratton, C. W., H. B. Hawley, T. A. Horsman, K. K. Tu, A. Ackley, N. K. Fernando, and M. P. Weinstein.** 1980. *Hemophilus influenzae* pneumonia in adults. Am. Rev. Respir. Dis. **121**:595–598.
40. **Teele, D. W., J. O. Klein, B. Rosner, L. Bratton, G. R. Fisch, O. R. Mathieu, P. J. Porter, S. G. Starobin, L. D. Tarlin, and R. P. Younes.** 1983. Middle ear disease and the practice of pediatrics. Burden during the first five years of life. J. Am. Med. Assoc. **249**:1026–1029.
41. **Tillotson, J. R., and A. M. Lerner.** 1968. *Haemophilus influenzae* bronchopneumonia in adults. Arch. Intern. Med. **121**: 428–432.
42. **Turk, D. C.** 1982. *Haemophilus influenzae*: epidemiology, immunology and prevention of disease. Elsevier, New York.
43. **Verghese, A., and S. L. Berk.** 1983. Bacterial pneumonia in the elderly. Medicine (Baltimore) **62**:271–285.
44. **Weinberg, G. A., M. S. Einhorn, A. A. Lenoir, P. D. Granoff, and D. M. Granoff.** 1987. Immunologic priming to capsular polysaccharide in infants immunized with *Haemophilus influenzae* type b polysaccharide-*Neisseria meningitidis* outer membrane protein conjugate vaccine. J. Pediatr. **111**:22–27.
45. **Weinberg, G. A., A. Ghafoor, Z. Ishaq, N. K. Nomani, M. Kabeer, F. Anwar, M. I. Burney, A. W. Qureshi, J. M. Musser, R. K. Selander, and D. M. Granoff.** 1989. Clonal analysis of *Hemophilus influenzae* isolated from children from Pakistan with lower respiratory tract infections. J. Infect. Dis. **160**:634–643.
46. **Weinberg, G. A., and D. M. Granoff.** 1988. Polysaccharide-protein conjugate vaccines for the prevention of *Haemophilus influenzae* type b disease. J. Pediatr. **113**:621–631.
47. **Weinberg, G. A., D. A. Towler, and R. S. Munson, Jr.** 1988. Lipoproteins of *Haemophilus influenzae* type b. J. Bacteriol. **170**:4161–4164.
48. **Zlotnick, G. W., V. T. Sanfilippo, J. A. Mattler, D. H. Kirkley, R. A. Boykins, and R. C. Seid, Jr.** 1988. Purification and characterization of a peptidoglycan-associated lipoprotein from *Haemophilus influenzae*. J. Biol. Chem. **263**:9790–9794.