INFECTION AND IMMUNITY, Oct. 1989, p. 3226–3229
0019-9567/89/103226-04$02.00/0

Vol. 57, No. 10

Immunological Responsiveness of Chinchillas to Outer Membrane and Isolated Fimbrial Proteins of Nontypeable *Haemophilus influenzae*

LAUREN O. BAKALETZ,* BARBARA M. TALLAN, WITOLD J. ANDRZEJEWSKI, THOMAS F. DEMARIA, AND DAVID J. LIM

Otological Research Laboratories, Department of Otolaryngology, College of Medicine, Ohio State University, Columbus, Ohio 43210

Received 17 April 1989/Accepted 29 June 1989

Thin, nonhemagglutinating fimbriae have been demonstrated on 100% of the clinical isolates of nontypeable *Haemophilus influenzae* recovered from children with chronic otitis media tested in this laboratory (L. O. Bakaletz, B. M. Tallan, T. M. Hoepf, T. F. DeMaria, H. G. Birck, and D. J. Lim, Infect. Immun. 56:331–335, 1988). Chinchillas with induced otitis media responded to this surface-located antigen of both the infecting and a heterologous strain. Antibodies were found in both serum and middle ear fluids.

Nontypeable (NT) *Haemophilus influenzae* is the leading causative agent of chronic otitis media with effusion (9). Unlike its type b counterpart, this organism lacks a strain marker, which has complicated the collection of epidemiological data (12, 19–21), development of a serotyping system (13), and progress in vaccine development. Recent studies have earmarked outer membrane proteins (OMPs) as significant targets of bactericidal antibody (5, 8, 17, 18). In particular, two OMPs, designated P2 and P6 (42 and 16.6 kilodaltons [kDa], respectively), have received a great deal of attention, and it is known that P2 is a porin protein (14). Both are being considered as potential vaccine components because of their surface location and, thereby, ease of accessibility to the immune system (4, 15–18).

Another polypeptide of potential immunological importance, which is the subject of this study, is bacterial fimbriae. Apicella et al. (1), have noted fimbriae in 3 of 15 strains of NT *H. influenzae* recovered from sputum and the eye of a patient with conjunctivitis. Our laboratory subsequently reported the presence of fimbriae on 100% of 60 clinical isolates of NT *H. influenzae* recovered from middle ear fluids (MEFs) of children with chronic otitis media with infusion (3). Fimbriated NT *H. influenzae* isolates were shown to adhere to human oropharyngeal cells. Fimbriae were reported to be 2.4 to 3.6 nm in width, and the degree of fimbriation did not correlate with the strength of the ability to hemagglutinate, nor did their presence confer the ability to hemagglutinate for all strains (3). It was of interest to us to attempt to isolate these structures, identify them within a total OMP preparation, and assess their immunological significance in a chinchilla model of otitis media.

(Part of this research was presented earlier [L. Bakaletz, Abstr. Assoc. Res. Otolaryngol. 1988, abstr. no. 278, p. 225].)

An inoculum of NT *H. influenzae* 1128 was prepared as described previously (3). A cohort of 10 healthy chinchillas was inoculated into the left superior bulla with 0.3 ml of a suspension (2×10^8 to 2×10^9 CFU/ml) of this moderately fimbriated strain. The chinchilla was selected because of its extensive use in studies of experimental otitis media; it has been shown (7a, 22) to be susceptible to pathogens associated with otitis media, and the large size of its bullae allows for the collection of sufficient volumes of effusions and bulla washes that can be worked with. Animals received 0.3 ml of sterile saline into the right superior bulla as a control. Each week for 10 weeks, serum was collected, an otoscopic exam was performed, and MEFs, if present, were collected from both ears or a bulla wash was performed as reported previously (2). A Gram stain and differential count were done on cytocentrifuged MEFs or bulla washes. In addition, we attempted to reisolate the infecting organism on chocolate agar plates. Recovered organisms were examined for the ability to hemagglutinate and for the presence of fimbriae, as previously reported (3).

Serum collected both pre- and postinoculation and effusions or bulla wash fluids were used in a Western blotting (immunoblotting) procedure versus whole OMP preparations from both the homologous and a heterologous strain (NT *H. influenzae* 86-028). A fimbrial preparation isolated from the infecting organism was also used in the Western blots. OMP preparations were made by a technique modified from that of Carlone et al. (7). Briefly, NT *H. influenzae* isolates were grown in brain heart infusion broth supplemented with NAD and hemin (Difco Laboratories, Detroit, Mich.). After 18 h of incubation, the broth was centrifuged at 4°C for 30 min ($3,000 \times g$). Bacterial pellets were suspended in 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.4; Sigma Chemical Co., St. Louis, Mo.) and sonicated (Artec Systems Corp., Farmingdale, N.Y.). Sonication was repeated for a total of six times for 10 s each time. Suspensions were centrifuged ($12,000 \times g$) for 5 min at 4°C, and the pellets were discarded. Supernatants were again centrifuged for 30 min, and the generated pellets were suspended in HEPES with equal volumes of 2% sarcosyl (Sigma) in 10 mM HEPES. Suspensions were held at room temperature for 30 min with intermittent shaking and were then centrifuged ($12,000 \times g$) for 30 min at 4°C. The resultant pellet was rinsed with double-distilled H_2O before it was resuspended in double-distilled H_2O and frozen (−20°C) until use.

OMP preparations were separated into individual proteins by electrophoresis in a 5 to 20% gradient gel after they were mixed with equal volumes of sample buffer and boiled for 1 min. Gels were run at a constant current of 60 mA for 70 min.

* Corresponding author.

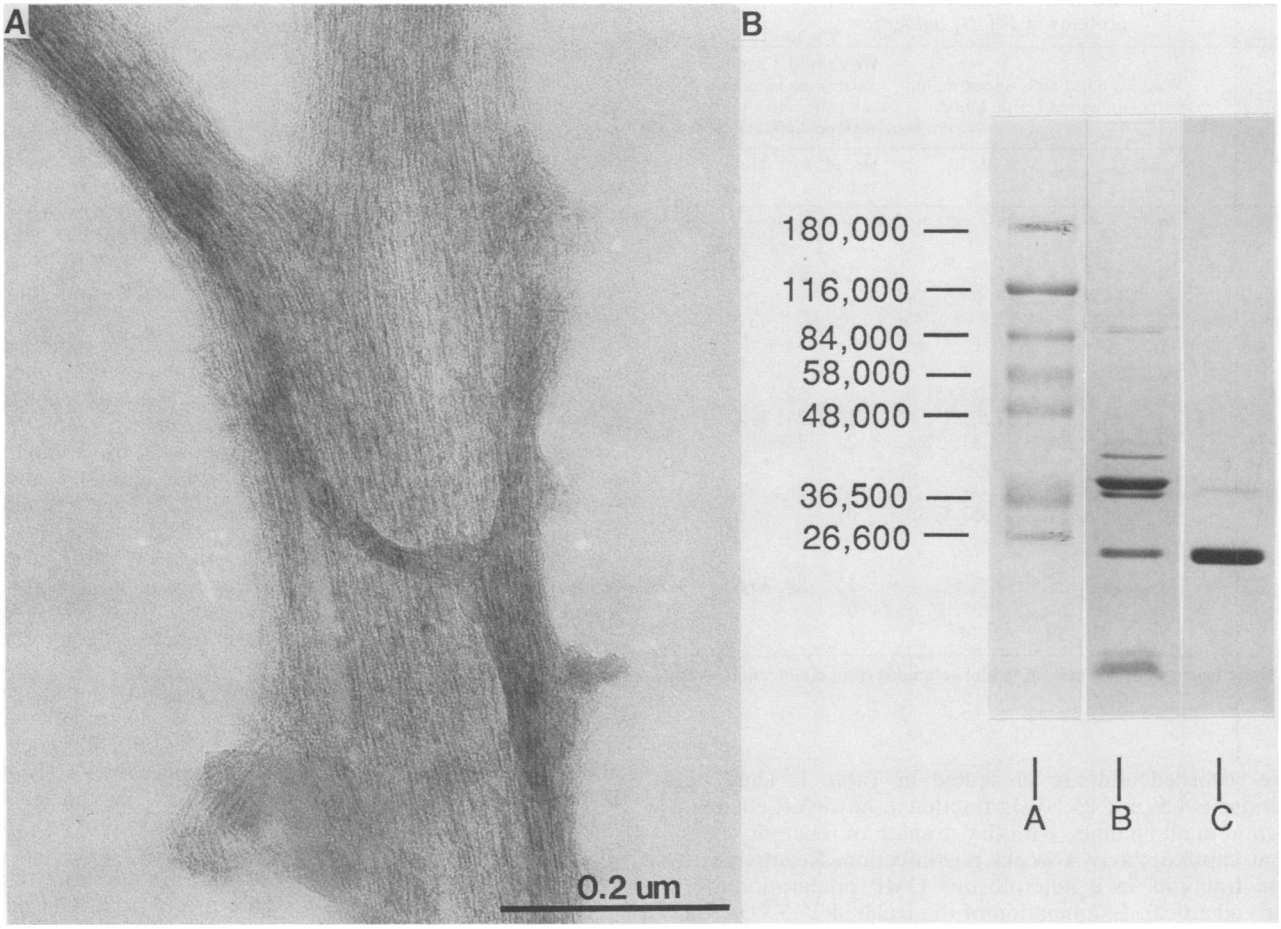

FIG. 1. (A) Transmission electron micrograph of negatively stained and isolated 25.5-kDa protein. Fimbriae line up as parallel bundles; extraneous outer membrane vesicles were not noted. (B) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of NT *H. influenzae* OMPs and isolated fimbrial proteins. Lanes: A, Prestained molecular weight markers; B, total OMP preparation from NT *H. influenzae*; C, isolated fimbrial preparation (25.5 kDa) plus minor contaminating OMP at approximately 38 kDa.

Prestained molecular weight markers (Sigma) were used to locate OMP fractions. The OMP fraction of interest was cut from the gel, and the protein was eluted by a technique developed in this laboratory. Briefly, strips of gel containing the 25.5-kDa fraction were homogenized and suspended in a portion of the running buffer (pH 8.3), followed by stirring overnight at 4°C. The resultant slurry was filtered to remove the gel matrix, and the filtrate was dialyzed against distilled water and concentrated to a small volume (Micro-ProDiCon; Bio-Molecular Dynamics, Beaverton, Oreg.). Fractions were stored frozen (−70°C) until use.

Western blots were performed by transfer of proteins to nitrocellulose (2 h, 60 V; Trans-Blot Cell; Bio-Rad Laboratories, Richmond, Calif.); washed; blocked with 2% bovine serum albumin in Tris-buffered saline containing 0.05% Tween 20 (TTBS; Sigma); and incubated with chinchilla serum, MEFs, or bulla washes (50 μl/10 ml of 1% bovine serum albumin in TTBS). Nitrocellulose sheets were washed with TTBS, incubated in horseradish peroxidase-protein A (40 μl/40 ml of 1% bovine serum albumin in TTBS; Zymed Laboratories, Inc., San Francisco, Calif.) for 1 h, washed with TTBS, and developed with 4-chloro-1-naphthol (Sigma). Development was stopped with distilled water, and membranes were air dried.

Tympanic membranes demonstrated signs of inflammation for up to 7 weeks postinfection, which correlated with the maximal time period in which NT *H. influenzae* could be recovered from MEFs. Organisms inoculated into the left bulla infected the right bulla, presumably by a transnasopharyngeal route. MEFs were collected from only 5 of 20 ears, even though organisms could be recovered from 8 ears. Differential counts revealed the expected transition from polymorphonuclear leukocyte-dominated effusions to those in which macrophages were predominant. All recovered NT *H. influenzae* isolates were able to hemagglutinate and bore fimbriae.

When assayed for preexisting or native immunological responsiveness to NT *H. influenzae* OMP, sera from a majority of preinoculated chinchillas demonstrated a weak but prevalent reaction in Western blots against total OMP preparations to three polypeptides (24, 38, and 39.5 kDa). This reactivity was augmented by the induced infection, as expected. Serum samples obtained postinfection demonstrated a significant response to several OMPs, as was expressed in two ways: (i) the appearance of bands not present in blots of serum samples obtained preinfection, and thereby representing a quantitative increase, or (2) an increase in the intensity of a band already demonstrated in blots of serum samples obtained preinfection, and thereby representing a qualitative increase. Both types of response

TABLE 1. Reactivity of paired chinchilla sera with electroblotted proteins of NT *H. influenzae*

Animal no. (weeks postinoculation)	Western blot bands appearing in postinfection serum blots[a]		Western blot bands demonstrating increased intensity when treated with postinfection serum	
	No. of bands	Mol wt (kDa)	No. of bands	Mol wt (kDa)
1	0		5	14.5, 24, 25.5, 38, 39.5
2	4	14.5, 31, 37.5, 42	3	24, 25.5, 38
3	7	14.5, 24, 25.5, 37.5, 38, 39.5, 42	0	
4	1	42	5	14.5, 24, 25.5, 38, 39.5
5	5	14.5, 25.5, 38, 42, 51	1	39.5
6	4	14.5, 25.5, 37.5, 51	2	24, 38
7	10	14.5, 24, 25.5, 31, 37.5, 39.5, 42, 50, 51, 112.5	2	38, 44
8	10	14.5, 24, 25.5, 37.5, 38, 39.5, 42, 44, 51, 112.5	0	
9	4	14.5, 25.5, 37.5, 51	2	38, 39.5
10	9	14.5, 24, 25.5, 31, 37.5, 38, 39.5, 42, 51	0	

[a] These bands were not present in blots obtained from preinfection serum samples.

were recorded and are presented in Table 1. Only two fractions (14.5- and 25.5-kDa fractions), however, elicited a reaction in all animals, with the strength of reactivity peaking at approximately 4 weeks postinfection. Reactivities to these fractions in a heterologous OMP preparation were nearly identical. Examination of the isolated 25.5-kDa fraction indicated that it was a relatively homogeneous preparation of fimbriae (Fig. 1). Fimbrial strands were not seen in preparations of an unrelated OMP (38 kDa) that was treated identically. When postinfection sera were blotted against enriched fimbrial preparations (Fig. 2), reactivity was again noted in 10 of 10 animals. MEFs and bulla washes were also assessed by the Western blot technique. Bulla washes from noninfected animals and MEFs induced by injection of keyhole limpet hemocyanin (2) were used as controls and gave no reaction (data not shown). Samples from ears of all infected animals showed responsiveness to the 25.5-kDa fraction of the total OMP preparation, with 10 of 10 infected ears and 6 of 10 control ears demonstrating a reaction. Only animals 4 and 7 (animals from which NT *H. influenzae* was recovered) demonstrated reactivity against the 25.5-kDa fraction in the isolated fimbrial preparations. Reactivity was moderate to strong in both ears of animal 7 and in the infected left ear of animal 4.

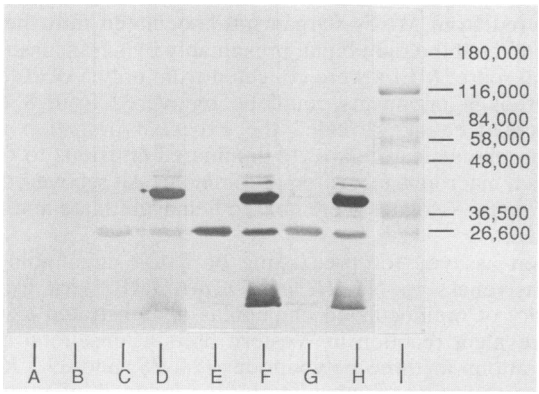

FIG. 2. Immunoblot assay of NT *H. influenzae* OMPs and isolated fimbrial subunits subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Lanes: B, D, F, and H, Whole OMP preparation; A, C, E, and G, isolated 25.5-kDa protein. The following lanes were assayed with the indicated sera or effusions from a chinchilla that had induced otitis media for 7 weeks: A and B, preinfection serum; C and D, postinfection serum; E and F, effusion collected from the right bulla; G and H, effusion collected from the left bulla. Lane I, Prestained molecular weight markers.

In summary, we attempted to further our understanding of the pathogenesis of NT *H. influenzae*-induced otitis media and the role of fimbriae in this disease. We isolated these fimbriae but still must purify them further to remove a contaminating band at 38 kDa. Regardless of this difficulty, polyacrylamide gel electrophoresis demonstrated that we successfully isolated, as a major band of 25.5 kDa, a fraction rich in fimbriae of the dimensions reported previously (3). Chinchillas with NT *H. influenzae*-induced otitis media responded serologically to this antigen, and antibody was found in both MEFs and bulla washes. Chinchillas also responded to the analogous protein in a heterologous strain of NT *H. influenzae*.

The potential value of this fimbrial protein as a vaccine component has yet to be demonstrated. We have, however, shown that this isolated preparation can inhibit adherence of NT *H. influenzae* to human oropharyngeal cells (L. O. Bakaletz, T. M. Hoepf, and D. J. Lim, submitted for publication). This protein may be analogous to that reported by LiPuma and Gilsdorf (11), which was present only on adherent isolates of type b *H. influenzae*. In addition, there have been two reports (6, 10) of the use of a purified pilus preparation from NT *H. influenzae* which successfully protected chinchillas against otitis media. These pili are morphologically and functionally distinct from those reported here, and yet both carry promise as potential human immunogens. While the protective effect of this preparation has yet to be shown, we have evidence that this protein exists in 12 of 12 isolates from humans with otitis media tested to date and that serological as well as local reactivity versus the 25.5-kDa protein can be readily documented (T. F. DeMaria et al., manuscript in preparation).

This study was supported, in part, by Public Health Service grant NSO8854 from the National Institute of Neurological and Communicative Disorders and Stroke.

We thank Toni Hoepf, Teresa Martin, Jeanne Furth, Valerie Jones, and Ilija Karanfilov.

LITERATURE CITED

1. **Apicella, M. A., M. Shero, K. C. Dudas, R. R. Stack, W. Klohs, L. J. LaScolea, T. F. Murphy, and J. M. Mylotte.** 1984. Fimbriation of *Haemophilus* species isolated from the respiratory tract of adults. J. Infect. Dis. **150**:40–43.
2. **Bakaletz, L. O., T. F. DeMaria, and D. J. Lim.** 1987. Phagocytosis and killing of bacteria by middle ear macrophages. Arch. Otolaryngol. **113**:138–144.
3. **Bakaletz, L. O., B. M. Tallan, T. M. Hoepf, T. F. DeMaria, H. G. Birck, and D. J. Lim.** 1988. Frequency of fimbriation of nontypable *Haemophilus influenzae* and its ability to adhere to chinchillas and human respiratory epithelium. Infect. Immun. **56**:331–335.
4. **Barenkamp, S. J.** 1986. Protection by serum antibodies, in experimental nontypable *Haemophilus influenzae* otitis media. Infect. Immun. **52**:572–578.

5. **Barenkamp, S. J., R. S. Munson, and D. M. Granoff.** 1982. Outer membrane protein and biotype analysis of pathogenic nontypable *Haemophilus influenzae*. Infect. Immun. **36**:535–540.
6. **Brinton, C. C., Jr., M. J. Carter, D. B. Derber, S. Kar, J. A. Kramarik, A. C.-C. To, S. C.-M. To, and S. W. Wood.** 1989. Design and development of pilus vaccines for *Haemophilus influenzae* diseases. Pediatr. Infect. Dis. J. **8**:554–561.
7. **Carlone, G. M., M. L. Thomas, H. S. Rumschlag, and F. O. Sottnek.** 1986. Rapid microprocedure for isolating detergent-insoluble outer membrane proteins from *Haemophilus* species. J. Clin. Microbiol. **24**:330–332.
7a. **Giebink, G. S., E. E. Payne, E. L. Mills, S. K. John, and P. G. Quie.** 1976. Experimental otitis media due to *Streptococcus pneumoniae*: immunopathogenic response in the chinchilla. J. Infect. Dis. **134**:595–604.
8. **Gnehm, H. E., S. I. Pelton, S. Gulati, and P. A. Rice.** 1985. Characterization of antigens from nontypable *Haemophilus influenzae* recognized by human bactericidal antibodies. J. Clin. Invest. **75**:1645–1658.
9. **Howie, V. M., J. H. Ploussard, and R. L. Lester.** 1970. Otitis media: a clinical and bacteriological correlation. Pediatrics **45**:29–35.
10. **Karasic, R. B., D. J. Beste, S. C.-M. To, W. J. Doyle, S. W. Wood, M. J. Carter, A. C.-C. To, K. Tanpowpong, C. D. Bluestone, and C. C. Brinton, Jr.** 1989. Evaluation of pilus vaccines for prevention of experimental otitis media caused by nontypable *Haemophilus influenzae*. Pediatr. Infect. Dis. J. **8**:S062–S065.
11. **LiPuma, J. J., and J. R. Gilsdorf.** 1988. Structural and serological relatedness of *Haemophilus influenzae* type b pili. Infect. Immun. **56**:1051–1056.
12. **Loeb, M. R., and D. H. Smith.** 1980. Outer membrane protein composition in disease isolates of *Haemophilus influenzae*: pathogenic and epidemiological implications. Infect. Immun. **30**:709–717.
13. **Murphy, T. F., and M. A. Apicella.** 1985. Antigenic heterogeneity of outer membrane proteins of nontypable *Haemophilus influenzae* is a basis for a serotyping system. Infect. Immun. **50**:15–27.
14. **Murphy, T. F., and M. A. Apicella.** 1987. Nontypable *Haemophilus influenzae*: a review of clinical aspects, surface antigens and the human immune response to infection. Rev. Infect. Dis. **9**:1–14.
15. **Murphy, T. F., and L. C. Bartos.** 1988. Human bactericidal antibody response to outer membrane protein P2 of nontypable *Haemophilus influenzae*. Infect. Immun. **56**:2673–2679.
16. **Murphy, T. F., and L. C. Bartos.** 1988. Purification and analysis with monoclonal antibodies of P2, the major outer membrane protein of nontypable *Haemophilus influenzae*. Infect. Immun. **56**:1084–1089.
17. **Murphy, T. F., L. C. Bartos, A. A. Campagnari, M. B. Nelson, and M. A. Apicella.** 1986. Antigenic characterization of the P6 protein of nontypable *Haemophilus influenzae*. Infect. Immun. **54**:774–779.
18. **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 nontypable *Haemophilus influenzae* as a target for human serum bactericidal antibody. J. Clin. Invest. **78**:1020–1027.
19. **Murphy, T. F., J. M. Bernstein, D. M. Dryja, A. A. Campagnari, and M. A. Apicella.** 1987. Outer membrane protein and lipooligosaccharide analysis of paired nasopharyngeal and middle ear isolates in otitis media due to nontypable *Haemophilus influenzae*: pathogenic and epidemiological observations. J. Infect. Dis. **156**:723–731.
20. **Paterson, A. J., K. F. Macsween, and T. H. Pennington.** 1987. *Haemophilus influenzae* subtyping by SDS-PAGE of whole cell polypeptides. Epidemiol. Inf. **99**:179–189.
21. **Spinola, S. M., J. Peacock, F. W. Denny, D. L. Smith, and J. G. Cannon.** 1986. Epidemiology of colonization by nontypable *Haemophilus influenzae* in children: a longitudinal study. J. Infect. Dis. **154**:100–109.
22. **Watanabe, N., B. R. Briggs, and D. J. Lim.** 1982. Experimental otitis media in chinchillas. I. Baseline immunological investigation. Ann. Otol. Rhinol. Laryngol. **91**(Suppl. 83):1–8.