Immunization with Outer Membrane Protein P6 from Nontypeable *Haemophilus influenzae* Induces Bactericidal Antibody and Affords Protection in the Chinchilla Model of Otitis Media

T. F. DeMARIA,* DEBRA M. MURWIN, AND EDWARD R. LEAKE

Otologic Research Laboratories, The Ohio State University
College of Medicine, Columbus, Ohio 43210

Received 13 March 1996/Returned for modification 1 May 1996/Accepted 4 September 1996

The role of nontypeable *Haemophilus influenzae* (NTHi) outer membrane protein (OMP) P6 in the pathogenesis of otitis media (OM) has not been defined. OMPs, fimbriae, pili, and lipooligosaccharide are several types of surface antigens of NTHi that are currently being evaluated as potential vaccine candidates. P6 is antigenically conserved among both nontypeable and type b *H. influenzae* strains and elicits bactericidal as well as protective antibodies; however, initial evaluation of a vaccine mixture of P6 combined with other NTHi OMPs failed to induce bactericidal antibody or protection in the chinchilla model of OM. We undertook an assessment of the ability of immunization with isolated P6 lipoprotein alone to confer protection. Chinchillas were immunized with P6 and challenged 10 days after the final immunization with either 3×10^3 CFU of NTHi delivered directly into the middle ear to induce OM or 5×10^8 CFU of NTHi delivered intranasally to establish nasopharyngeal colonization. All immunized animals responded with elevated serum titers of anti-P6 antibody, which also demonstrated bactericidal activity against homologous as well as a heterologous NTHi isolate. By 14 days post-transbullar challenge, the number of chinchillas with middle ear fluid and the incidence of NTHi culture-positive middle ear fluids were reduced 48 and 51%, respectively, in the P6-immunized chinchillas relative to the sham-immunized cohort. Nasopharyngeal colonization levels were comparable in the two cohorts. These data demonstrate that active immunization with P6 results in the production of NTHi-specific bactericidal antibody in the chinchilla and also affords a reduction in the incidence of NTHi-induced OM; however, parenteral immunization does not appear to affect the extent or duration of nasopharyngeal colonization by NTHi.

Nontypeable *Haemophilus influenzae* (NTHi) strains have emerged in recent years as significant pathogens, particularly with regard to otitis media (OM) and other respiratory tract diseases, and may cause more invasive disease than previously recognized. NTHi strains have been associated with an increasing number of mucosal diseases, including OM, sinusitis, bronchitis, pneumonia, and conjunctivitis (22, 33), and may play a role in recurrent acute tonsillitis and tonsillar hypertrophy (30). It is now recognized that in adults, the spectrum of diseases caused by NTHi includes exacerbation of chronic bronchitis (22) and chronic obstructive pulmonary disease (13).

NTHi strains are an extremely diverse and heterogeneous group of bacteria. Initiatives to identify surface antigens of *H. influenzae* which are conserved among NTHi strains and elicit protective antibody are being actively pursued to identify potential components for a vaccine. P6 is a highly conserved, surface-exposed peptidoglycan-associated lipoprotein present in the outer membrane of all strains of NTHi and *H. influenzae* type b (Hib) tested to date (14, 23, 25). Antibodies directed against P6 have been shown to be protective against Hib disease in the infant rat bacteremia model (20). The ability of anti-P6 antibody to protect against experimental NTHi disease, however, has not been established, although enhanced respiratory clearance of NTHi has recently been reported fol-

lowing mucosal immunization of rats with P6 (17). An evaluation of mixtures of purified *H. influenzae* outer membrane proteins (OMPs) Hi-PAL (P6), recombinant PCP, and OMPe (P4) in the chinchilla model of OM indicated that immunization with these three OMPs mixed together did not protect against NTHi-induced OM (12). Moreover, detectable anti-NTHi bactericidal antibodies were not produced. These data were surprising since previous reports by these investigators had established that mixtures of antisera directed against these OMPs show synergistic killing of both Hib and NTHi cells in vitro bactericidal assays (6, 9, 11). Therefore, it was anticipated that synergy might also be demonstrated in vivo after immunization with a mixture of all three lipoproteins. Perhaps, as the authors themselves suggested in their discussion, these proteins when mixed could have formed a complex which induced conformational changes resulting in altered immunogenicity. Ongoing investigations in our laboratory with P6 prompted us to investigate whether immunization with P6 alone might induce biologically active antibody in the chinchilla and whether this antibody is protective against nasopharyngeal colonization and NTHi-induced OM.

Transbullar challenge as used in this study and by Green and coworkers (12) does not allow for an evaluation of the potential protective role of anti-P6 antibody during the entire spectrum of OM pathogenesis, which includes colonization of the nasopharynx with NTHi, retrograde ascension of the eustachian tube, and finally invasion and multiplication in the middle ear cleft. An intranasal (i.n.) cohort was included as part of this study to provide an initial assessment of whether systemic immunization with P6 alters the extent and duration of colonization of the nasopharynx. Our data indicate that active

* Corresponding author. Mailing address: Otologic Research Laboratories, The Ohio State University College of Medicine, Room 4331 UHC, 456 West 10th Ave., Columbus, OH 43210. Phone: (614) 293-8103. Fax: (614) 293-5506. Electronic mail address: tdemaria@magnus.acs.ohio-state.edu.

immunization with P6 induces anti-NTHi bactericidal antibody in the chinchilla, which indicates that the previously reported inability of this species to produce bactericidal antibodies is not due to the innate inability of the chinchilla to produce antibodies against bactericidal epitopes of NTHi protein antigens. Immunization with P6 does not affect the level or duration of nasopharyngeal colonization after i.n. challenge; however, it does afford protection during experimental OM, as evidenced by a reduced incidence and duration of culture-positive middle ear fluids (MEFs) after transbullar challenge of the middle ear with NTHi.

(This work was presented in part at the 95th General Meeting American Society for Microbiology, Washington, D.C., 1995; the Eighteenth Midwinter Research Meeting, Association for Research in Otolaryngology, 1995; and the Sixth International Symposium on Recent Advances in Otitis Media, 1995.)

MATERIALS AND METHODS

Bacterial strains. The NTHi strains used in this study were 1128 and 86-028NP. Both strains (originally isolated from children with OM) have been used extensively by our laboratory and described previously (29, 31). NTHi cells were grown in brain heart infusion broth (BBL Becton Dickinson, Cockeysville, Md.) supplemented with hemin (10 µg/ml; Sigma, St. Louis, Mo.) and NAD (10 µg/ml; Sigma) at 37°C in a humidified atmosphere of 5% CO₂ and 95% air.

Animals. A total of 42 chinchillas (*Chinchilla laniger*, 350 to 650 g) free of middle ear disease, as determined by otoscopy and tympanometry, were used for these studies. The chinchilla was chosen because of its widely accepted use as an experimental model of human OM and because of previous reports regarding immunization of this species with P6 (12).

Isolation of P6. P6 was isolated by a modification of methods previously described by Murphy et al. (23, 24). Briefly, overnight cultures of NTHi strain 86-028NP were harvested by centrifugation at 3000 × g for 30 min at 4°C, washed with phosphate-buffered saline (PBS), and resuspended in 1% sodium dodecyl sulfate (SDS)–0.1 M Tris–0.5 M NaCl–0.1% β-mercaptoethanol (pH 8.0) (buffer B). The cells were disrupted by sonication (Artek Sonic Dismembrator model 150; Artex Systems Corporation, Farmingdale, N.Y.) and incubated at 37°C for 30 min in a shaking water bath. The slurry was clarified by centrifugation at 21,000 × g at room temperature for 30 min. The supernatant was discarded, and the pellets were resuspended in a total of 250 ml of buffer B containing 10 µg of RNase A (Sigma) per ml. The sonication, centrifugation, and resuspension in buffer B were repeated three times, except after the third centrifugation, when the pellets were resuspended in 250 ml of buffer B without RNase A. To solubilize P6, the pellets were resuspended in 36 ml of 0.01 M Tris–0.15 M NaCl (pH 7.4) (buffer A) and incubated at 65°C for 30 min in a shaking water bath at 75 rpm, sonicated for 15 s on setting 60 at 5-min intervals, and centrifuged at 100,000 × g for 60 min at 30°C. The supernatant containing the P6 was dialyzed against buffer A to remove SDS and concentrated approximately 10-fold by using a Micro-Pro DiCon membrane (10,000-molecular-weight exclusion; Spectrum, Houston, Tex.). The protein concentration of the P6 preparations was determined as described by Murphy et al. (23). The endotoxin concentration of the P6 was assessed by means of a chromogenic *Limulus* amoebocyte lysate assay (Whittaker M.A. Bioproducts, Walkersville, Md.).

SDS-PAGE. Isolated P6 and whole cell preparations were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) in a 5 to 20% continuous gradient minigel (0.01% [wt/vol] SDS) run at a 30 mA per gel for approximately 1 h as described previously (29). Gels were stained with Coomassie brilliant blue or silver stained for lipopolysaccharide as described by Tsai and Frasch (32).

Western blot (immunoblot) assay. Western blot analyses were performed by transfer of proteins to nitrocellulose (1.5 h, 100 V; Trans-Blot cell; Bio-Rad Laboratories, Richmond, Calif.), washed, blocked with 2% bovine serum albumin (BSA) in Tris-buffered saline containing 0.05% Tween 20 (TTBS), and incubated with chinchilla pre- or postimmunization serum or a P6-specific monoclonal antibody, 7F3 (kindly provided by Timothy Murphy, Buffalo, N.Y.) which has been characterized and described in detail previously (27). Additional blots were probed with monoclonal antibody 3D2, a lipooligosaccharide (LOS)-specific monoclonal antibody, also provided by Timothy Murphy, to rule out LOS contamination of the isolated P6 lipoprotein. This antibody has been described in detail previously (24). Nitrocellulose sheets were washed with TTBS. Blots probed with chinchilla serum were incubated with horseradish peroxidase-protein A (50 µl/10 ml of 1% BSA in TTBS; Zymed Laboratories, Inc., San Francisco, Calif.) for 1 h, washed with TTBS, and developed with 4-chloro-1-naphthol (Sigma). Blots probed with monoclonal antibodies 7F3 and 3D2 were incubated with a polyclonal horseradish peroxidase-conjugated goat antiserum specific for mouse immunoglobulins G, A, and M (20 µl/10 ml of 1% BSA in TTBS; Zymed Laboratories). Development was stopped with distilled water, and membranes were air dried.

Chinchilla immunization. Forty-two chinchillas were separated into two co-

horts of 21 animals each. Twenty-one chinchillas were each immunized by the subcutaneous injection of 50 µg of P6 in 500 µl of PBS emulsified 1:1 with 500 µl of complete Freund's adjuvant (Difco, Detroit, Mich.). Three additional 50-µg booster immunizations in incomplete Freund's adjuvant were given at 3-week intervals. Blood for sera were obtained before and 1 week after the primary immunization, boost, and final immunization. The second cohort of chinchillas was sham immunized with PBS diluent emulsified in Freund's adjuvant.

ELISA for P6. Chinchilla sera were evaluated by means of an enzyme-linked immunosorbent assay (ELISA) using isolated P6 as the antigen as previously described (26) with the exception that horseradish peroxidase-protein A (Zymed Laboratories) was used to detect bound chinchilla antibody since no chinchilla-specific reagents are commercially available. Color was developed by the addition of 2,2-azino-d (3-ethylbenzthiazoline) sulfuric acid (ABTS) substrate. One hundred microliters of a solution containing 5 or 0.5 µg of P6 per ml was added per well. Assays were performed in duplicate, and the endpoint titers are reported as the reciprocal of the highest dilution giving an optical density at 405 nm (OD₄₀₅) of ≥0.1. Controls included wells in which coating buffer was used in place of serum and wells without P6 (antigen). Preimmune sera were always assayed concurrently with immune sera from the same animal, and a high-titered serum diluted 1/50,000 from animal 26 was run with each assay and served as an internal standard.

Bactericidal assays. Bactericidal assays were performed against NTHi strain 86-028NP, the strain also used for preparing P6 lipoprotein, by a modification of the method of Green et al. (11, 12). A limited number of sera were also assayed against NTHi strain 1128, a heterologous strain isolate. A 120-µl aliquot of sera from sham-immunized or pre- and post-P6-immunized, prechallenge chinchillas was heated at 56°C for 30 min to inactivate complement and placed on ice. Sera were diluted serially twofold, in duplicate, in cold 2× PCM buffer (PBS containing 0.15 mM CaCl₂, 0.5 mM MgCl₂, and 0.5% BSA [fraction V; Sigma]) (1). Sixty-microliter aliquots of the serum dilutions were prepared aseptically and maintained on ice. NTHi strain 86-028NP cells were washed and adjusted to an OD₄₉₀ of 0.2 (7.6 × 10⁷ CFU/ml) and diluted to approximately 6 × 10³ CFU/ml of PCM buffer. The NTHi suspension was centrifuged and resuspended to the same concentration by the addition of 650 50% hemolytic complement units per ml of PCM buffer. Guinea pig complement (Calbiochem, La Jolla, Calif.) with a specific activity of 1,000 50% hemolytic complement units/ml was used as the complement source. A 60-µl volume of the cells per complement suspension was added to each 60-µl serum dilution. All reactions were performed in duplicate, and the mixtures were incubated for 1 h at 37°C in a shaking water bath. The reaction mixtures were returned to ice, and a 50-µl aliquot from each reaction mixture was plated on chocolate agar. After overnight incubation, the plates were counted and bactericidal activity was determined. The bactericidal titer was defined as the reciprocal of the highest dilution of antiserum capable of killing greater than 50% of the bacteria compared with controls not containing antibodies. Additionally, four sera were also assayed, using a heterologous strain (1128) as the test organism for the bactericidal assay.

Absorption of bactericidal activity. Selected sera were incubated with 30 µg of P6 (250 µg/ml) at 4°C for 18 h before the bactericidal assays were performed. Controls consisted of serum incubated with diluent without P6.

Intranasal challenge and assessment nasopharyngeal colonization of immunized chinchillas. The i.n. cohort was included to provide an initial assessment of whether systemic immunization with P6 alters the extent and duration of colonization of the nasopharynx. While colonization by NTHi is an important step in OM pathogenesis, it requires analysis separate from OM, for i.n. inoculation of the chinchilla with NTHi strains alone typically induces only inconsistent tympanic membrane inflammation and does not result in culture positive OM with MEF or sustained presence of the organism (2). Ten days postimmunization, seven chinchillas from each of the sham- and P6-immunized cohorts were challenged i.n. with 500 µl of a suspension containing approximately 5 × 10⁸ CFU of NTHi per ml. The inoculum was divided between nares. Nasopharyngeal lavage was performed every 4 to 7 days for semiquantitative assessment of CFU of NTHi per milliliter of lavage fluid as previously described (29, 31). Additionally, the chinchillas were evaluated as described below for the transbullar inoculation.

Transbullar middle ear challenge and induction of OM. Ten days after final immunization, seven chinchillas from each of the sham- and P6-immunized cohorts were challenged with 3 × 10⁵ CFU of NTHi strain 1128 inoculated directly into the superior aspect of the left cephalid bulla to induce OM. The bullae were vented with a sterile 26-gauge needle placed near the inoculation site. The chinchillas were evaluated for tympanic membrane inflammation and middle ear pressure changes daily for 28 days by means of otoscopy and tympanometry. Observations were performed blindly, and tympanic membrane inflammation was rated 0 to 4+ as previously described (29). Normal chinchilla middle ear pressure was considered to be between –60 and +40 daPa (28). Epitympanic taps of the inferior bulla were performed every 4 to 7 days postchallenge for the semiquantitative determination of viable CFU of NTHi per milliliter of MEF. Briefly, for this procedure, the chinchilla was anesthetized with ketamine and the fur from the area over the superior aspect of the cephalid bulla was removed. The area was swabbed with betadine, and a 22-gauge needle, affixed to a tuberculin syringe, was inserted to the level of the inferior bulla. A 26-gauge needle was used as a vent, and up to 300 µl of MEF was gently aspirated from each ear. This technique is well established (4, 29) and allows for a reliable assessment of the concentration of bacterial pathogenesis in the MEF.

FIG. 1. Lanes: A, Coomassie brilliant blue-stained SDS-PAGE-separated molecular mass markers (in daltons); B, total OMP preparation from NTHi 86-028NP; C, isolated P6 from strain 86-028NP.

Finally, seven animals in each cohort were dedicated to a serial histopathological analysis and were not subject to epitympanic taps. The middle ear mucosa from the inferior bulla was recovered and fixed for subsequent evaluation of hematoxylin-eosin-stained tissue sections.

Statistical analyses. Cohorts within each study were compared for relative number of chinchillas with disease, and the numbers of NTHi culture-positive MEFs were compared by chi-square analysis with Yates correction for continuity. A *P* value of ≤ 0.05 was accepted as the minimum level for significance.

RESULTS

Isolation of P6. P6 resolved as a single band with a molecular mass of approximately 16 kDa (Fig. 1, lane C). The P6-specific monoclonal antibody 7F3 recognized P6 both in the total OMP preparation (Fig. 2, lane C) and as an isolated OMP (Fig. 2, lane D) in Western blot analysis. LOS was found to be present only at a concentration of 0.3 ng/mg of protein and not detectable by silver stain or with monoclonal antibody 3D2 in Western blots (data not shown).

Serum ELISA P6 antibody response. Preimmunization serum antibody reciprocal titers from the sham- and P6-immunized chinchillas were 90 and 50, respectively. After three immunizations with 50 μ g of P6 each, the reciprocal titer increased to 125,000 (Table 1). The sham-immunized cohort titers increased to approximately three times the preimmunization values. A subsequent and final booster immunization resulted in a twofold increase in titers of the immunized cohort; the sham-immunized cohort titers declined to preimmunization levels.

Western blot assay. Sera obtained from P6- and sham-immunized animals were used to detect P6 by Western analysis of total OMP preparations and isolated P6. The 16-kDa bands in both the total OMP and isolated P6 lipoprotein preparations showed reactivity (Fig. 3, lanes C and D, respectively) when

FIG. 2. Lanes: A, Coomassie brilliant blue-stained SDS-PAGE-separated molecular mass markers (in daltons); B, total OMP preparation (lane B); C and D, Western blots of total OMP preparation from NTHi 86-028NP (lane C) and isolated P6 lipoprotein from strain 86-028NP (lane D) probed with P6-specific monoclonal antibody 7F3.

TABLE 1. Serum antibody titers against P6

Immunogen ^a	Mean serum antibody titer ^b against P6 $\pm$ SD		
	0 ^c	3	4
P6	50 $\pm$ 60	120,000 $\pm$ 750	250,000 $\pm$ 150,000
PBS	90 $\pm$ 100	300 $\pm$ 300	90 $\pm$ 100

^a In each case, 21 chinchillas were tested.

^b Reciprocal of the highest dilution with an OD₄₀₅ of ≥ 0.1 .

^c Number of immunizations.

probed with sera from animals immunized with P6. Isolated P6 also exhibited a second reactive band, when probed with polyclonal chinchilla sera, with an approximate molecular mass of 24 kDa (lane D) that is a putative dimer (25) which was not present in the total OMP preparation. The diffuse second band above P6 in lane C is presumably due to reactivity with LOS present in the total OMP preparation. The preimmune sera and sera from sham-immunized animals showed minimal reactivity with approximate molecular masses of 30 kDa (Fig. 3, lane E and F, respectively).

Bactericidal activity. Minimal bactericidal activity against NTHi was present in 2 of the 21 preimmunization sera. By the third immunization, bactericidal activity was present in the sera of all chinchillas in the immunized cohort (Fig. 4). Reciprocal titers ranged from 2 to 64. Sham immunization did not induce any detectable bactericidal antibody activity (data not shown). Those sera assayed against the heterologous NTHi strain 1128 demonstrated the same titer as when assayed against the homologous strain 86-028NP (data not shown). A single overnight absorption of the sera with 30 μ g of P6 lipoprotein either completely removed or reduced the bactericidal activity of each serum up to fourfold (Table 2).

Transbullar challenge with NTHi. As expected, by 3 days postchallenge, approximately 50% of the chinchillas demonstrated evidence of OM. By 7 days postchallenge, a majority of the transbullar-injected chinchillas in both the sham-immunized and immunized cohorts had NTHi culture-positive OM. Numbers of CFU of NTHi per milliliter of MEF for individual ears are shown in Table 3. As depicted, six of the seven sham-immunized animals showed signs of OM by day 7. Eight of the fourteen ears within this cohort contained MEF, and all eight MEFs grew pure cultures of NTHi. At day 7, there were minor differences in the number of chinchillas with MEF and the incidence of NTHi culture-positive MEFs between the sham and immune cohorts. Bilateral involvement indicated by right ear involvement was evident in two chinchillas in each of the

FIG. 3. Western blot of total OMP preparation from 86-028NP (lanes C and E) or isolated 86-028NP P6 lipoprotein (lane D and F), using chinchilla anti-P6 antisera (lanes C and D) or sera from sham-immunized chinchillas (lanes E and F). The reactive band in lane D with a molecular mass of approximately 24 kDa is a presumed dimer formed by isolated P6 lipoproteins; the diffuse second band in lane C is presumably due to reactivity with LOS present in the total OMP preparation. Molecular masses are indicated in daltons.

FIG. 4. Serum bactericidal activity in chinchillas before and after four immunizations. Each data point represents the average titer from samples run in duplicate for each time period.

respective cohorts. By 14 days postchallenge, however, the number of chinchillas with MEF and the incidence of NTHi culture-positive MEFs were reduced by 48 and 51%, respectively, in the P6-immunized chinchillas compared with the sham-immunized cohort. The rates of clearance of bacteria from the tympanic cavity were different between the two cohorts, as evidenced by epitympanic tap culture data (Table 3). Five of six chinchillas (83%) in the sham-immunized cohort exhibited signs of OM, compared with two of five (40%) animals in the P6-immunized cohort. Seven MEFs were recoverable from sham-immunized animals, compared with three in the immunized cohort (Table 3). Whereas these trends towards a reduction in both incidence of disease and duration of culture-positive MEFs are clinically apparent, they were not found to be statistically significant because, in part, of the small sample size. By 21 days postchallenge, signs of OM were resolving in both cohorts.

Tympanic membrane inflammation and middle ear under pressures. Immunization with P6 did not protect against tympanic membrane inflammation post-transbullar challenge. The tympanic membrane inflammation scores were identical between the sham-immunized and immunized cohorts. Maximal inflammation (3 and 4+) scores were observed between 9 to 15 days postinoculation (data not shown). Similarly, both the immunized and sham-immunized cohorts exhibited abnormal tympanograms (data not shown).

Induction of nasopharyngeal colonization. Nasopharyngeal lavage fluids obtained on day 3 postchallenge from both the sham- and P6-immunized chinchillas yielded counts in the range of 10^2 to 10^6 CFU/ml for both groups. Through day 21, nasopharyngeal colonization data were comparable between cohorts and did not indicate that immunization with P6 affected nasopharyngeal colonization by NTHi (data not shown).

DISCUSSION

Unlike Hib, for which a highly successful vaccine is available, there is no vaccine to protect against NTHi-induced disease, nor is the immune response induced against Hib cross-protective. Efforts to develop a vaccine for disease caused by the unencapsulated NTHi strains have focused on OMPs, fim-

brae, pili, and LOS (19). A number of the OMPs of NTHi, including P6, have been investigated extensively as potential components for the development of a vaccine for disease induced by these organisms. Unlike P6, many of these other surface-exposed antigens, however, are not conserved among NTHi strains, and data indicate that immunity to these bacteria may be strain specific (14, 25).

Data generated in this study showed that immunization with P6 lipoprotein alone induces high titers of serum anti-P6 antibody in the chinchilla. Moreover, the sera from P6-immunized animals are bactericidal for NTHi in vitro. In addition, immunization affords protection, as demonstrated by a reduced incidence and duration of culture-positive MEFs in the chinchilla model of OM. Previously, Green et al. (12) reported an evaluation of the protective efficacy of P6 which indicated that P6, when administered as part of a vaccine mixture of three NTHi OMPs, i.e., PCP-PAL, Hi-PAL (P6), and OMPe (P4), elicited high serum ELISA titers to each respective component but did not induce bactericidal antibody or confer protection in the chinchilla model of NTHi-induced OM. The use of an OMP mixture for immunization in contrast to the isolated P6 lipoprotein used in the present study may account, in part, for these conflicting data. As originally suggested by Green and coworkers, the three proteins comprising the vaccine mixture could have formed a complex resulting in altered protein conformation and masking of protective epitopes. The fact that isolated P6 lipoprotein has a propensity to form dimers (21), as evidenced by the Western blot analysis with chinchilla antisera, supports the possibility that protein complex formation occurred. Other notable differences between the studies include the use of different P6 isolation procedures; the P6 used by Green and coworkers was derived from a Hib strain, whereas the P6 lipoprotein used in this study was isolated from an NTHi strain; the complements used for the bactericidal assays in the two studies were also from different species. Although Green et al. did not detect any bactericidal antibody in sera from animals immunized with the purified OMP mixtures, the use of agammaglobulinemic human serum as the complement source by these investigators did permit demonstration of serum bactericidal activity from animals immunized with whole formalin-fixed NTHi cells or OMP fractions. Therefore, the chinchilla does have the ability to produce antibodies against NTHi bactericidal epitopes, which suggests that the formulation of the immunogen is an important determinant for the induction of bactericidal antibody. Finally, the

TABLE 2. Absorption of bactericidal antibody activity with P6

Specimen ^a	Titer ^b	
	Unabsorbed	Absorbed ^c
36T	16	0
42Q	16	0
39Q	16	0
41T	8	0
31Q	16	0
31T	32	16
32T	64	16
28T	32	8
34T	32	16
29T	16	8
38T	64	16

^a T, three immunizations; Q, four immunizations.

^b Reciprocal of the highest dilution of serum killing >50% of NTHi test strain 1128.

^c Serum incubated with 30 μ g of P6 (250 μ g/ml) at 4°C 18 h prior to assay.

TABLE 3. Viable bacteria in epitympanic tap fluids after transbullar challenge with NTHi 1128^a

Group	3 days		7 days		14 days		21 days	
	Ear	CFU/ml	Ear	CFU/ml	Ear	CFU/ml	Ear	CFU/ml
Sham immunized	28L	8.2×10^7	41R	1.3×10^6	32L	5.1×10^7	24L	8.3×10^3
	36L	2.8×10^8	32L	7.3×10^8	32R	5.7×10^8		
	32L	2.0×10^6	32R	3.6×10^7	16L	2.4×10^5		
			37L	3.4×10^6	16R	3.1×10^3		
			16R	6.3×10^6	28L	8.0×10^4		
			16L	6.0×10^7	24L	1.2×10^5		
			28L	4.4×10^6	36L	3.8×10^7		
			36L	1.6×10^8				
	(3/14) ^b	(3/3) ^c	(8/14)	(8/8)	(7/12)	(7/7)	(1/10)	(1/1)
	3/7		6/7		5/6		1/5	
Immune	15L	2.4×10^6	15R	1.9×10^6	7L	2.4×10^5	7L	1.2×10^6
	2L	6.1×10^4	2L	3.8×10^3	3L	2.1×10^5		
	10L	3.8×10^7	7L	6.7×10^6	3R	3.8×10^6		
	34L	1.6×10^7	3L	1.8×10^6				
			3R	1.0×10^5				
			34L	4.9×10^5				
	(4/14)	(4/4)	(6/14)	(6/6)	(3/10) ^c	(3/3)	(1/8)	(1/1)
No. of chinchillas with OM/no. tested	3/7		5/7		2/5 ^d		1/4	

^a L, left ear; R, right ear. Two animals in each cohort demonstrated bilateral OM.^b Number of MEFs/total no. of ears.^c Number culture positive/no. of MEFs. Reduction versus sham at 14 days.^d Reduction versus sham at 14 days.

NTHi strain used by Green and coworkers to challenge the chinchillas appears to be highly virulent. An inoculum of only 10 CFU of NTHi per ear was used to induce OM. A review of the literature indicates that typical doses required to induce OM by the transbullar route average approximately 10^3 CFU per ear to consistently induce OM in the chinchilla (7).

It is well established that antibodies directed against P6 are bactericidal for both Hib and NTHi in vitro (11, 24). Moreover, antibody to P6, derived from human serum, has been shown to be bactericidal for all *H. influenzae* strains tested to date (10). A study by Yamanaka and Faden (34) which characterized the serum antibody response of children with OM to various NTHi OMPs suggests that certain children, who are classified as otitis prone, do not respond well to P6, whereas non-otitis-prone individuals are more likely to develop a significant antibody response to this OMP. Principal uncertainties remain, however, concerning the ability of young children to respond to P6 and whether protection is afforded to the host by anti-P6 antibodies. Our data from the chinchilla model of OM corroborate previous reports that immunization with P6 induces bactericidal antibody.

Active immunization of the chinchilla with formalin-fixed NTHi whole cells or total OMP preparations has been previously shown to provide a significant degree of protection during NTHi-induced OM when the rate of recovery of NTHi from the middle ear is used as an outcome measure (4). Immunized chinchillas exhibited recovery rates of 45 to 50% of control values in these studies. Data from the present study indicate that by 14 days postchallenge, immunization with isolated P6 lipoprotein alone promotes an accelerated clearance of bacteria from the chinchilla tympanic cavity. Culture-positive MEFs were reduced by 51% in the immunized cohort. The data indicate that antibody directed against certain P6 epitopes accelerates the clearance of NTHi from the middle ear and thereby modulates the immune response in favor of the host. Reformulation of the immunizing preparation or use of different routes of immunization may enhance the clearance rate even further.

The impact of immunization on signs and symptoms of OM is less clear from the present study. The tympanic membrane inflammation outcome data as well as the tympanometry data failed to demonstrate any differences between the sham-immunized and P6-immunized cohorts. These findings may be due, in part, to the fact that considerable tympanic membrane inflammation has also been shown to be induced by NTHi peptidoglycan (18) and/or endotoxin (8) remaining in the middle ear after the viable bacteria have been eliminated. Green and coworkers also noted a lack of protection when otoscopy was used as an outcome measure (12).

The i.n. cohort was included in this study to provide an initial assessment of whether systemic immunization with P6 alters the extent and duration of colonization of the nasopharynx. Intranasal inoculation is not an acceptable method for producing NTHi-induced otitis media. Intranasal inoculation of the chinchilla with NTHi strains alone typically induces only inconsistent tympanic membrane inflammation and does not result in culture-positive OM with MEF or sustained presence of the organism (2). Alternatively, transbullar challenge of the middle ear directly does not allow for an evaluation of the potential protective role of anti-P6 antibody during the entire spectrum of OM pathogenesis, which includes the initial interaction, which is colonization of the nasopharynx with NTHi; retrograde ascension of the eustachian tube; and finally invasion and multiplication in the middle ear cleft. While colonization is an important step in OM pathogenesis, it requires analysis separate from OM unless one uses a combined adenovirus-NTHi inoculation of the chinchilla (31). Data from this study indicate that parenteral immunization with P6 does not alter the extent or duration of nasopharyngeal colonization. Anti-P6 antibody levels were not measured as part of this study to determine whether the i.n. challenge is effective in inducing sufficient inflammation and transudation of serum antibody into the nasopharynx.

Delineation of the specifics of a protective immune response induced by P6 or other NTHi antigens requires the use of a suitable experimental model of NTHi disease, of which there

are few. Various aspects of NTHi pulmonary clearance have been studied in mice (15) and rats (5, 17). These pulmonary models, however, are limited to studying clearance and augmentation thereof by immunization due to the fact that the inocula are eliminated from the lungs with no evidence of sustained NTHi multiplication. The chinchilla model of NTHi-induced OM is, however, well established and the only available model for NTHi disease whereby each stage of the host-parasite interaction can be studied from initial adherence to resolution of disease. There is evidence of bacterial multiplication, extensive histopathological damage, and induction of a specific immune response (3, 4, 7, 16). Our findings indicate that the efficacy of P6 as a potential NTHi vaccine component can and should be further evaluated effectively with the chinchilla model of OM.

In conclusion, active immunization with P6 induces anti-NTHi bactericidal antibody in the chinchilla, which indicates that the previously reported inability of this species to produce bactericidal antibodies is not accurate. The chinchilla has the innate ability to produce bactericidal antibody against epitopes protein antigens of NTHi. Moreover, immunization with P6 affords a level of protection during experimental OM, as evidenced by a trend toward a reduced incidence and duration of culture-positive MEFs. P6 therefore warrants continued investigation as a component of a vaccine for NTHi disease.

ACKNOWLEDGMENTS

We thank Valerie Jones for generation of tables and figures and Jan Webb for manuscript preparation.

This study was supported in part by research grant 5 R01 DC 00090-24 from the National Institute on Deafness and Other Communication Disorders, National Institutes of Health.

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