INFECTION AND IMMUNITY, May 1993, p. 1950–1957
0019-9567/93/051950-08$02.00/0

Vol. 61, No. 5

Evaluation of Mixtures of Purified *Haemophilus influenzae* Outer Membrane Proteins in Protection against Challenge with Nontypeable *H. influenzae* in the Chinchilla Otitis Media Model

BRUCE A. GREEN,[1]* MARY E. VAZQUEZ,[1] GARY W. ZLOTNICK,[1] GERI QUIGLEY-REAPE,[1] J. DOUGLAS SWARTS,[2] ISRAEL GREEN,[2] JAMES L. COWELL,[1] CHARLES D. BLUESTONE,[2] AND WILLIAM J. DOYLE[2]

Lederle-Praxis Biologicals, Inc., 211 Bailey Road, West Henrietta, New York 14586-9728,[1] and Department of Pediatric Otolaryngology, Children's Hospital of Pittsburgh, University of Pittsburgh Medical School, Pittsburgh, Pennsylvania 15213[2]

Received 26 October 1992/Accepted 3 March 1993

Nontypeable *Haemophilus influenzae* (NTHi) is one of the leading causative agents of bacterial otitis media, and no vaccine has been shown to be effective against it. Three outer membrane lipoproteins of NTHi have been investigated extensively and are leading candidates for inclusion in a vaccine against this organism. Hi-PAL (P6), recombinant PCP (rPCP), and *e* (P4) proteins are antigenically conserved among NTHi strains and elicit bactericidal and protective antibodies. A genetic fusion of the rPCP and Hi-PAL proteins has also been reported. Mixtures of these proteins were used for active immunization experiments in the chinchilla model of otitis media. Chinchillas were immunized either with a mixture of all three lipoproteins or with the mixture of rPCP-PAL hybrid plus *e* protein. When these animals were challenged with a NTHi strain injected directly into the middle ears, no protection from infection or disease, as measured by otoscopy, was observed in either group. However, effusion and inflammation measured by tympanometry were significantly reduced in animals immunized with the three lipoproteins. Animals that had been immunized with either whole NTHi cells or total outer membranes and then challenged with the homologous strain were significantly protected from both infection and disease, as determined by tympanometry and otoscopy. Unlike other animal antisera, chinchilla antisera against the purified proteins had no bactericidal activity against NTHi but did fix complement on the cell surface. Thus, the chinchilla immune responses to mixtures of these lipoproteins differ from the immune responses observed in other animal species. Further evaluation of these proteins for their vaccine potential remains to be done.

Nontypeable *Haemophilus influenzae* (NTHi) is a leading cause of human otitis media and upper respiratory infections including pneumonia and sinusitis (3, 29). Current vaccines against *H. influenzae* are effective only against *H. influenzae* strains possessing the type b capsular polysaccharide (Hib). Efforts to develop vaccines against NTHi have focused on outer membrane proteins (OMPs) and pili, with several laboratories reporting biologically active antibodies directed against the OMPs of NTHi. Antibodies against P1 (30), P2 (15), *e* protein (P4) (12), Hi-PAL (P6) (14, 27), and recombinant PCP (rPCP) (5) are bactericidal against NTHi strains in vitro. However, the biologic activity reported against the P1 and P2 proteins appears to be strain specific, which severely limits the potential usefulness of these proteins as components in a vaccine for NTHi. At least three OMPs capable of eliciting biologically active antibodies, the Hi-PAL, PCP, and *e* proteins, have been shown to be antigenically conserved (5, 12, 14, 26, 28). Further, anti-Hi-PAL and anti-*e* protein sera are capable of protecting infant rats from meningitis caused by Hib (12, 14, 24).

Several animal models of otitis media have been developed to evaluate the efficacies of therapeutic agents for otic infections or the prophylactic potentials of candidate components for otitis media vaccines. Two of these models are the chinchilla (10) and rat (17) models of otitis media. The rat model has been used for pneumococcal otitis media (17), while the chinchilla model has been used for prophylaxis of NTHi (23, 32) and pneumococcal (33) infections and for the development of vaccines against these organisms (1, 8, 18, 30, 33). Work with the chinchilla model has demonstrated that viable bacteria are not required for the symptoms of acute otitis media with effusion (7, 11), indicating that biologically active cell walls and/or endotoxin may be important mediators of the clinical symptoms of otitis in the chinchilla. Previous experiments with NTHi demonstrated that passive immunization with chinchilla antisera directed against whole NTHi cells (1) or rabbit antisera against the LKP pili of NTHi (18) can protect chinchillas from a direct challenge with homologous NTHi strains. Karasic et al. (18) reported that active immunization with partially purified pili preparations could protect chinchillas from a direct challenge to the middle ear. Recently, Pelton et al. (30) reported preliminary evidence that active immunization with a preparation of OMP P1 was capable of protecting chinchillas against direct challenge with the homologous NTHi strain.

Since antisera against Hi-PAL, rPCP, and *e* protein are bactericidal against NTHi, and anti-Hi-PAL and anti-*e* protein are protective against experimental Hib meningitis, we initiated experiments to determine the abilities of these proteins and of the recently reported rPCP–Hi-PAL hybrid protein (6) to protect chinchillas from direct challenge with NTHi after active immunization. The results reported in this

* Corresponding author.

manuscript demonstrate that when given as a mixture, the three OMPs and/or the hybrid protein and *e* protein do not elicit detectable biologically active antibodies in chinchillas and are not protective against experimental otitis media after direct challenge.

MATERIALS AND METHODS

Bacterial strains. *H. influenzae* strains used in this study were NTHi 860295 obtained from C. Bluestone (Pittsburgh, Pa.) and Hib Eagan (14). NTHi 860295 has been previously used as a challenge strain in the middle ears of chinchillas (4, 18). Hib laboratory strain Eagan was the source of Hi-PAL and *e* OMPs. *Escherichia coli* JM103 (36) carrying plasmid pPX163 (5) and JM103 (pPX512) (6) have been previously described. *E. coli* strains were grown in LB medium (22) containing ampicillin (Sigma Chemical Co., St. Louis, Mo.) (100 μg/ml), and *H. influenzae* strains were grown in brain heart infusion (Difco Laboratories, Detroit, Mich.) supplemented with hemin (Sigma) (10 μg/ml) and NAD (Sigma) (2 μg/ml) (BHI-XV) at 37°C.

Preparation of vaccines. Whole formalin-fixed NTHi cells were prepared from mid-log-phase NTHi 860295 cells (4) grown in BHI-XV to an optical density at 490 nm (OD_{490}) of 1.0. Cells were washed in PCM buffer (phosphate-buffered saline [PBS] containing $CaCl_2$ and $MgCl_2$ [14]) and resuspended in PCM buffer to a concentration of 2×10^9 cells per ml. Formalin was added to a final concentration of 0.4%, and the cells were incubated for 1 h at room temperature. Formalin was removed by washing the cells in PCM buffer, and the cells were resuspended to a final concentration of 2×10^9 cells per ml in sterile saline. Outer membrane fractions of strain 860295 were prepared essentially by the method of Zlotnick et al. (37). The outer membranes were resuspended in sterile saline to a concentration of 1.3 mg/ml.

Purified OMPs of *H. influenzae* were prepared by previously described methods (5, 6, 12, 37). Hi-PAL and *e* proteins were isolated from Hib Eagan; rPCP was isolated from *E. coli* JM103(pPX163), and the genetic fusion of rPCP and Hi-PAL was purified from *E. coli* JM103(pPX512). All proteins were resuspended in 0.85% NaCl containing 0.01% thimerosal at the final purification step.

Determination of protein levels. Protein levels were determined by the method of Lowry et al. (21) as modified by Peterson (31) or by the bicinchoninic acid (Pierce Biochemicals, Chicago, Ill.) method according to the manufacturer's directions.

Experimental plan. The animal study was a randomized, blind, controlled study of the efficacy of active immunization in the prevention of acute otitis media caused by NTHi using the chinchilla model. A total of 130 adult chinchillas weighing between 400 and 500 g were purchased and randomly assigned to one of five equal ($n = 26$) experimental groups. All animals were kept in quarantine for a period of 2 weeks to acclimate to the laboratory. A 3-ml blood sample was obtained by cardiac puncture for the baseline antibody assay. Five days after the animals were bled, they were injected intramuscularly with the coded vaccine appropriate to the assigned group. The immunizations were repeated twice as described below, and 15 days after completion of the injection sequence, the animals were again bled for the antibody response assay. Five days later, the middle ears of all animals were bilaterally challenged with approximately 10 CFU of NTHi. Every other day postchallenge for 14 days, cultures were taken from the left middle ear and examined for the presence of NTHi and the right middle ear was examined by otomicroscopy and tympanometry for evidence of acute otitis media. On day 14, cultures from the right ears were also taken and the animals were sacrificed by barbiturate overdose. All examiners were unaware of the group assignment of the animals. The study was reviewed and approved by the Animal Research and Care Committee at the Children's Hospital of Pittsburgh.

Immunization of chinchillas. Chinchillas were anesthetized with ketamine HCl (20 mg/kg of body weight) as previously described (4) prior to all operative procedures. Animals were immunized intramuscularly with 10^7 formalin-fixed whole cells per animal, 25 μg of total OMPs, or mixtures containing 75 μg of total protein (either 50 μg of rPCP-PAL hybrid plus 25 μg of *e* protein or 25 μg each of Hi-PAL, rPCP, and *e* protein). All vaccines were emulsified 1:1 in incomplete Freund's adjuvant (Difco) in a total volume of 0.2 ml. Two booster immunizations were given at monthly intervals.

NTHi challenge of chinchillas. The NTHi challenge strain, 860295, was passed through the bullae of a chinchilla as previously described (18), and aliquots of log-phase cells (10^7 CFU/ml) were frozen at −70°C in Trypticase soy broth (TSB)-20% glycerol. Aliquots were thawed and used to prepare inocula by dilution into PBS (pH 7.2) to the proper concentration. Immunized chinchillas were held for 5 days after the prechallenge bleeding to allow recovery from anesthesia. Chinchillas were anesthetized with ketamine (20 mg/kg), and the area over the superior bullae was shaved. A 22-gauge needle attached to a 1-ml tuberculin syringe containing the inoculum was introduced into the middle ear via puncture of the superior bullar skin and bone. Then, 0.1 ml of inoculum containing approximately 10 organisms was instilled into the middle ear, and the needle was removed. Bacterial challenges were performed bilaterally.

On day 2, the animals were anesthetized with ketamine and the area over the left superior bullae was swabbed with Betadine. A 2-mm-long incision was made in the soft tissue over the left epitympanum, and a small hole was made through the bullar bone with a scalpel. A culture from the middle ear was taken as described below, and the incision was closed with a single surgical staple. Cultures of the left middle ear were taken every other day through day 14. On those days, the bullar skin was swabbed and the surgical staple was removed. The bullar defect was exposed with a hemostat, and a calcium alginate swab moistened in TSB and mounted on a flexible aluminum shaft was introduced into the left middle ear space to come into contact with the hypotympanum mucosa. The swab was removed and then streaked directly onto a chocolate agar plate (BBL) which was incubated in 5% CO_2 for 24 to 48 h at 37°C. The plates were examined at 24 and 48 h for growth typical of NTHi colonies, and X- and V-factor growth requirements were ascertained by the paper strip (BBL) technique. The skin was closed with a surgical staple. On day 14, cultures from the right middle ears were taken as described above.

At the time of left middle ear culture and while the animals were anesthetized, a diagnosis of acute otitis media with effusion was made for the right ears by both otomicroscopy and tympanometry. For otomicroscopy, the chinchilla was positioned on an operating table with the ipsilateral ear up and a speculum was introduced into the ear canal. The speculum was positioned to allow for visualization of the tympanic membrane under low power (6× magnification) using a Zeiss otomicroscope. A positive diagnosis of acute otitis media was made if the tympanic membrane was red and inflamed, had observable air fluid levels, was bulging with obvious yellow fluid, or was perforated with drainage to

the external canal. Tympanometry was then performed with a clinical instrument (Teledyne automatic impedance meter TA-7A) with standard adult probe tips. For each test, volume, compliance, and middle ear pressure were recorded. A measured volume of greater than 1.5 ml and a compliance of 0 ml was interpreted as perforation of the tympanic membrane. Middle ear fluid was diagnosed if the compliance was less than 0.7 ml. Middle ear inflammation was diagnosed if the recorded pressure was less than −100 daPa or if an effusion was diagnosed to be present.

For the primary outcome measure of bacterial colonization, animals in all groups were assigned to one of two categories. The animals were considered to be culture positive if the NTHi challenge strain was recovered on any day prior to and including the day of analysis. The animals were considered culture negative if bacteria were not recovered on any day. These assignments were made for all surviving animals at days 4 and 14. On these days, the differences between groups were evaluated for statistical significance by the chi-square test with Yates correction for continuity.

For the secondary outcomes related to otoscopy and tympanometry, the average number of abnormal observations per animal for each measure was calculated. This measure was considered to be a continuous variable, and between-group differences in these measures were evaluated for statistical significance by the two-tailed Student *t* test. All comparisons were made with the saline placebo group. Statistical significance was evaluated at $\alpha = 0.05$.

ELISAs against purified proteins. Enzyme-linked immunosorbent assays (ELISAs) using Hi-PAL, rPCP, and *e* protein were done as previously described (5, 12, 14) except that recombinant protein G labeled with alkaline phosphatase (Zymed Laboratories, So. San Francisco, Calif.) was used to detect bound chinchilla antibodies, since no reagents specific for chinchilla antibodies are available commercially. This reagent was found to be nonreactive with the protein-coated wells and bound to chinchilla immunoglobulins in test reactions. Reactions were allowed to continue, and titers were calculated as previously described (12).

Whole-cell and complement fixation ELISAs. Whole NTHi 860295 cells were prepared as described above except after fixation, the bacteria were suspended in PBS without formaldehyde and diluted to an OD_{620} of 0.2. Cells (75 μl) were then added to the wells of 96-well Immulon 1 microtiter plates (Dynatech Laboratories, Chantilly, Va.) and dried overnight at 37°C. The plates were blocked with PBS–0.1% gelatin for 1 h at 37°C and washed with PBS–0.1% Tween 20 in a Titertek 120 microplate washer (Flow Laboratories, McLean, Va.). Samples (100 μl) of antiserum diluted in PBS containing 0.15 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.1% gelatin, and 0.3% Tween 20 (PCM-GT) were added to the wells, and the plates were incubated for 2 h at 37°C. After the plates were washed, bound antibodies were detected with alkaline phosphatase-conjugated recombinant protein G and *para*-nitrophenylphosphate (Sigma) in diethanolamine buffer. Endpoint titers were determined as the reciprocal of the highest dilution giving an OD_{405} of 0.1. These conditions were shown to be specific for surface-exposed epitopes (data not shown). All samples were run in duplicate and the titers were averages of the two optical densities. Paired serum samples were run in parallel, e.g., preimmune sera was always run with immune sera from the same group.

For the complement fixation assay, bound antibodies were reacted with 100 μl of guinea pig complement (Rockland, Gilbertsville, Pa.) per well diluted 1:80 in PCM-GT for 1 h at 37°C. The plates were then washed, and bound complement was detected with 100 μl of alkaline phosphatase-conjugated rabbit anti-guinea pig C3 (Kirkegaard & Perry Laboratories, Gaithersburg, Md.) per well diluted 1:500 in PCM-GT. Endpoint titers were determined as above. Control reactions with rabbit and mouse anti-Hi-PAL (13, 14) and normal rabbit sera demonstrated that bactericidal antibodies fixed complement to the cell surface and that non-surface-reactive antisera did not fix complement (data not shown).

Bactericidal assays. Bactericidal assays against NTHi 860295 were performed as described previously (12) with the following modifications. Samples (15 μl) of antiserum, heated to 56°C for 20 min to remove complement activity and diluted 1:10 in PCM buffer, were added to the first well of a 96-well microtiter plate (Becton Dickinson Labware, Oxnard, Calif.). Twofold serial dilutions of antisera in PCM buffer were placed in the remaining wells, and the plates were held on ice. The complement source (agammaglobulenemic human serum provided by J. Leddy, Rochester, N.Y.) was absorbed against the nontypeable strain by the method of Green et al. (12). Bacterial cells grown to an OD_{490} of 1.0 were diluted 1:80,000 in PCM buffer containing the complement source (at a dilution of 1:2.5) and 0.5% bovine serum albumin (largely immunoglobulin free; Sigma). The microtiter plates were removed from ice, and 15-μl portions of the bacterial suspension containing complement were added to each well to initiate reactions. The plates were incubated at 37°C for 45 min, and 10 μl of each reaction mixture was then plated on BHI-XV. After overnight incubation at 37°C, colonies were counted to determine bactericidal titer (the reciprocal of the highest dilution of antiserum capable of killing greater than 50% of bacteria compared with controls not containing antibodies). To be considered significant, immune sera titers must be fourfold (i.e., two wells) greater than preimmune sera titers.

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a minigel system (70 by 100 mm) (Bio-Rad, Richmond, Calif.) by the method of Laemmli (20). Samples were reduced with β-mercaptoethanol or dithiothreitol in sample preparation buffer and boiled for 5 min. Gels were run at a constant voltage of 150 V. Separated proteins were detected by staining with Coomassie brilliant blue G-250 (Sigma). Lipooligosaccharide was detected in SDS-polyacrylamide gels by the silver staining method of Tsai and Frasch (35). Proteinase K was obtained from Sigma.

RESULTS

SDS-PAGE analysis of vaccines. Vaccines were prepared as described above and analyzed by SDS-PAGE. Samples of each purified protein and outer membranes as noted in Fig. 1 were loaded into the lanes of a 15% acrylamide gel and electrophoresed. Proteins were stained with Coomassie brilliant blue. Purified Hi-PAL and rPCP show single bands at approximately 15 kDa as expected (Fig. 1, lanes 3 and 4). The purified *e* protein migrates as a single band at approximately 28 kDa (lane 2), as previously described (12). The purified rPCP-PAL hybrid protein has a major band at 30 kDa (lane 5), with some minor bands below representing breakdown products, as determined by Western blot (immunoblot) analysis (data not shown). The outer membrane vaccine shows multiple bands typical of the complex mixture of proteins. Lipooligosaccharide contamination of the purified proteins was judged by silver staining of SDS-polyacrylamide gels by the method of Tsai and Frasch (35) and found to be less than 10 ng/μg of protein (data not shown).

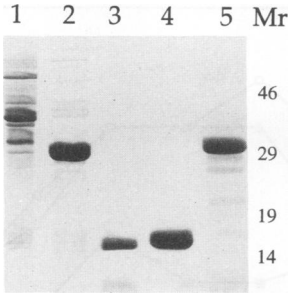

FIG. 1. SDS-PAGE analysis of vaccines used in this study. Samples of the vaccines were run on a SDS–15% polyacrylamide gel and stained with Coomassie brilliant blue. Lanes: 1, 20 μg of outer membranes from NTHi 860295; 2, 16 μg of native *e* protein; 3, 12 μg of rPCP; 4, 20 μg of native Hi-PAL; 5, 28 μg of rPCP-PAL hybrid protein. The approximate sizes (in kilodaltons) of the molecular size standards (Mr) are shown to the left of the gel.

Protection from infection and disease. On the day of challenge, the sample sizes for the five groups had been reduced by attrition to 22, 20, 21, 20, and 21 for groups vaccinated with whole cells, outer membranes, Hi-PAL, rPCP, *e*-protein mixture, the rPCP-PAL hybrid and *e* protein mixture, and placebo, respectively. During the postchallenge follow-up period, the respective sample sizes were further reduced to 20, 18, 19, 20, and 18 animals by day 14. There were no significant differences in mortality between groups during the pre- or postchallenge period.

The effects of active immunization on the three outcome measures of experimental NTHi otitis media described in Materials and Methods were examined. Table 1 reports the number of animals in each of the five experimental groups from which NTHi was recovered on days 4 and 14. On day 4, data are available for all challenged animals and show that NTHi was recovered from all negative-control animals (immunized with saline). In contrast, a significant degree of protection was documented for animals immunized with either whole cells ($P = 0.0073$) or outer membranes ($P = 0.0253$); NTHi was recovered from only 45 and 50% of these positive-control groups. However, animals immunized with the mixture of Hi-PAL, rPCP, and *e* protein or with the mixture of rPCP-PAL and *e* protein were not protected and had NTHi recovery rates of 86 and 100%, respectively. These results were essentially unchanged when the recovery rates of NTHi on any study day in animals surviving the 14-day study period are considered (Table 1). The results from analysis of the left ears were further reinforced by the single-point culture results for the right middle ear on day 14, namely, 1 of 20 (5%), 6 of 18 (33%), 14 of 19 (74%), 13 of 20 (65%), and 10 of 18 (56%) ears were positive for NTHi in animals immunized with the mixture of whole cells, membranes, Hi-PAL, rPCP, and *e* protein, with the mixture of the rPCP-PAL fusion protein and the *e* protein, and with saline, respectively. Past studies (8, 32) have documented differences in culture-positive ears for violated ears and nonviolated ears. Nonviolated ears tend to have a lesser frequency of culture positivity. However, while the absolute percentages are not in agreement (left ear versus right ear), the between-treatment-group differences are consistent with respect to relative frequencies for both ears. It is thought that the repeated middle ear cultures may damage the mucosa and thus limit the effectiveness of bacterial clearance mechanisms.

Otomicroscopy showed signs of acute otitis media on at least 1 day of follow-up in the majority of animals in all groups. Specifically, of the animals surviving for the 14-day follow-up period, signs of otitis media were recorded for the right ears of 70 and 89% of animals immunized with whole cells and membranes and in 100% of the right ears of the animals immunized with Hi-PAL, rPCP, and *e* proteins, with rPCP-PAL hybrid and *e* proteins, or with saline. The frequencies of abnormal otomicroscopic observations for the right ears of surviving animals on each study day are shown in Fig. 2. Approximately 40% of the ears in both experimental vaccine groups and in the control (saline) group were abnormal by day 2. In these three groups, the frequency of abnormal otoscopic observations increased by day 4 to between 60 and 90% and remained relatively constant for the duration of the follow-up period in the groups given saline and rPCP-PAL hybrid plus *e* protein. The group immunized with the mixture of the three lipoproteins showed a slightly more rapid clearing of otitis. In comparison, the frequencies of abnormal signs for the two positive-control groups (whole cells and OMPs) showed delayed onset, lesser magnitude, and for whole-cell immunizations, resolution by study day 14. The average number of abnormal observations per animal was 5.4 ± 1.8 for the animals immunized with saline. No significant difference was observed for this variable between the negative-control group and the animals immunized either with the three lipoproteins (4.3 ± 2.2) or with the rPCP-PAL hybrid plus *e* protein (4.6 ± 1.9). However, animals immunized with the whole cells (2.0 ± 1.8) or membranes (3.2 ± 2.2) had significantly less abnormal observations than the saline group.

In animals surviving to day 14, right middle ear effusion was diagnosed by tympanometry on at least one occasion in all animals in the groups immunized with saline or the mixture of rPCP-PAL hybrid plus *e* protein, and in the

TABLE 1. Culture-positive otitis media in actively immunized chinchillas challenged with NTHi 860295

Immunogen[a]	Day 4		Day 14 (left ear)		*P* value[d]
	No. of animals[b]	No. of culture-positive animals[c]	No. of animals[b]	No. of culture-positive animals[c]	
Saline (control)	21	21	18	18	
Whole NTHi cells	22	10	20	9	0.0073
Outer membranes	20	10	18	9	0.0253
Hi-PAL, rPCP, and *e* protein	21	18	19	18	0.8185
rPCP-PAL hybrid and *e* protein	20	20	20	20	1

[a] Animals were immunized with three doses of vaccines in incomplete Freund's adjuvant.
[b] Total number of animals surviving in each group at this time point.
[c] Total number of animals in each group that survived and had positive middle ear cultures.
[d] *P* value of the number of animals that were culture positive by day 4 in each test group versus the value for the saline (control) group by chi-square analysis.

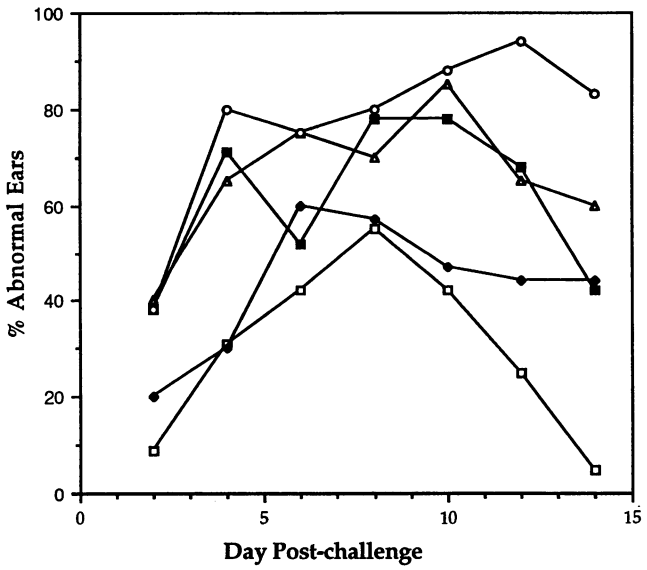

FIG. 2. Time course of middle ear disease in chinchillas challenged with NTHi, as determined by otoscopy (see text). The percentage of abnormal ears in each treatment group is shown versus time postchallenge. Symbols: □, whole cells; ◆, outer membranes; △, rPCP-PAL fusion protein plus *e* protein; ■, Hi-PAL, rPCP, and *e* protein; ○, saline (control).

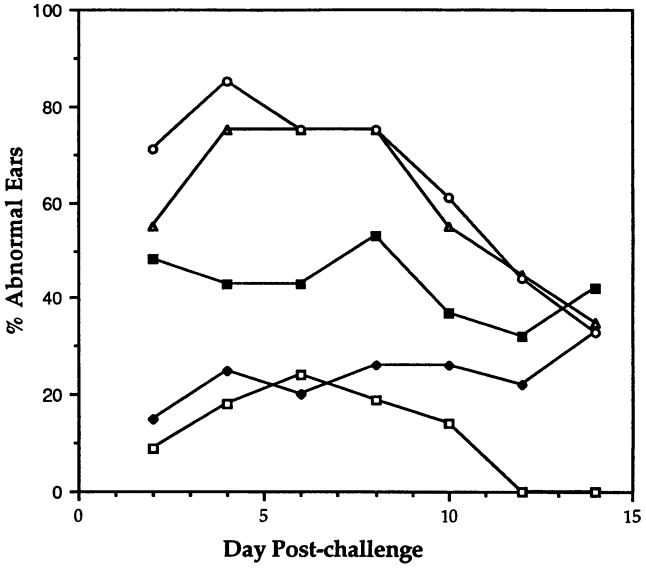

FIG. 3. Otitis media with effusion, as measured by tympanometry in chinchilla right middle ears after challenge with NTHi. The frequency of abnormal ears for each treatment group is plotted against time postchallenge. Normal middle ears are defined as having a compliance of 0.7 ml. Symbols: □, whole cells; ◆, outer membranes; △, rPCP-PAL fusion protein plus *e* protein; ■, Hi-PAL, rPCP, and *e* protein; ○, saline (control).

majority (15 of 19) of animals immunized with the mixture of Hi-PAL, rPCP, and *e* protein. In contrast, only seven ears in each of the two positive-control groups were diagnosed as having an effusion at any time during the follow-up period. The frequencies by group of tympanometrically diagnosed right middle ear effusion for surviving animals on each study day are shown in Fig. 3. By day 4, approximately 20% of the right ears of animals in the two positive-control groups had effusion. For the group immunized with membranes, this value was maintained throughout the follow-up period. However, effusion had resolved in all right ears of animals immunized with whole cells by study day 12. Between 70 and 90% of the animals immunized with saline or the rPCP-PAL hybrid plus *e* protein had effusion of the right ears by day 4, with both groups showing a gradual decrease in the frequency of effusion to approximately 35% by day 14. The frequency of right middle ear effusion for the group immunized with Hi-PAL, rPCP, and *e* protein was relatively constant over time and intermediate between that of the positive-control groups and the saline-immunized group. The average number of observations per animal for which effusion was diagnosed was not significantly different between the control (saline) group (4.4 ± 1.5) and the group immunized with rPCP-PAL hybrid plus *e* protein (4.2 ± 2.2). However, compared with the negative-control group, all other groups showed a significant reduction in the average number of observations with effusion. Specifically, the average values were 0.4 ± 0.7, 1.7 ± 1.3, and 2.9 ± 2.5 for the groups immunized with whole cells, outer membranes, and the three lipoproteins, respectively.

The majority of right ears in surviving animals in all groups evidenced middle ear inflammation on at least 1 day, as diagnosed by tympanometry. Specifically, all animals in groups immunized with saline and with rPCP-PAL hybrid plus *e* protein, 18 of 19 animals immunized with Hi-PAL, rPCP, and *e* protein, 18 of 20 animals immunized with whole cells, and 15 of 18 animals immunized with outer membranes were diagnosed with middle ear inflammations. The frequency of right middle ear inflammation on each study day was relatively constant for the two experimental vaccine groups, the control (saline) group and the group immunized with outer membranes for days 2 through 10. All groups showed a slight decrease in the frequency of inflammation between days 10 and 14. Specifically, approximately 90, 80, 70, and 50% of animals in groups immunized with saline, with rPCP-PAL fusion plus *e* protein, with Hi-PAL, rPCP, and *e* protein, and with outer membranes had inflammations on days 2 through 10. In contrast, inflammation was diagnosed in approximately 65% of the ears of animals immunized with whole cells on day 2 but declined in frequency to only 14% by day 10 and to 10% by day 14. As with the average number of observations with effusion, the groups immunized with the three lipoproteins, whole cells, and outer membranes had significantly less observations of inflammation than the saline control group.

Characterization of antisera from immunized chinchillas. With incomplete Freund's adjuvant (IFA) used as an adjuvant, chinchillas responded to all vaccines with high ELISA titers against both purified proteins (Table 2) and whole cells (Table 3). The saline group (control) sera had some antibodies that reacted with the three purified OMPs tested (Table 2), but not with whole cells (Table 3). This result may be due to infection of some of the animals in this group with bacteria containing cross-reacting antigens, as proteins which cross-react with conserved non-surface-exposed regions of these purified OMPs have been detected in *E. coli* (data not shown). In any event, these antibodies did not react with the NTHi cell surface (Table 3) or confer protection in chinchillas.

In general, the response to Hi-PAL, PCP, and *e* protein in the animals immunized with whole cells was much lower

TABLE 2. ELISA titers of pooled chinchilla serum samples against purified proteins

Vaccine	Sera	ELISA titer against:		
		Hi-PAL	rPCP	*e* protein
Whole cells	Preimmune	122	1,295	<100
	Immune	12,062	2,225	1,118
Membranes	Preimmune	100	1,439	<100
	Immune	131,698	37,099	318,524
Hi-PAL, rPCP, and *e* protein	Preimmune	1,781	1,739	<100
	Immune	661,063	3,143,500	2,878,110
rPCP-PAL and *e* protein	Preimmune	3,282	3,746	<100
	Immune	130,609	3,078,460	2,551,130
Saline (control)	Preimmune	100	4,477	<100
	Immune	5,799	45,910	28,877

than in all of the other test groups. This result was an expected one, since these three proteins are minor OMPs, probably consisting of much less than 1% of the total cell protein. Thus, the amount of Hi-PAL, PCP, and *e* protein in the immunizing dose of cells (10^7) is very low. Not surprisingly, the response of the whole-cell group to whole cells is respectably high (Table 3). The response to the rPCP portion of the rPCP-PAL hybrid protein was much higher than that against the PAL portion. Antibody response to *e* protein was very high in all samples tested, with the exception of the whole-cell vaccine. When measured against whole cells (Table 3), the vaccines all elicited high levels of surface-reactive antibodies, with the mixture of rPCP-PAL hybrid plus *e* protein giving the lowest level. These results indicate that levels of surface-reactive serum antibodies do not correlate with the level of protection.

Chinchilla antisera were tested for bactericidal activity against the challenge strain in the in vitro bactericidal assay as described above. Anti-whole cell and anti-outer membrane sera were bactericidal against the homologous NTHi 860295 strain used as the vaccine source (Table 4). However, sera from animals immunized with the Hi-PAL, rPCP, and *e* proteins or with the rPCP-PAL hybrid protein and *e* protein had no detectable bactericidal activity against NTHi 860295. Negative-control sera (from animals immunized with saline in IFA) had no bactericidal activity, as the preimmune sera

TABLE 3. Whole-cell and complement fixation ELISA titers of pooled chinchilla antisera

Immunogen	Titer in:			
	Whole-cell ELISA[a]		Complement fixation ELISA[b]	
	Preimmune	Immune	Preimmune	Immune
Formalin-fixed NTHi	132	365,402	<100	1,121
Outer membranes	108	3,646,803	<100	15,008
Hi-PAL, rPCP, and *e* protein	<100	869,878	<100	4,004
rPCP-PAL hybrid and *e* protein	231	261,056	<100	2,217
Saline (control)	168	161	<100	<100

[a] ELISA titer measured against formalin-fixed NTHi 860295 cells.
[b] ELISA titer measuring guinea pig C3 fixed by chinchilla antibodies on the surfaces of NTHi 860295 cells.

TABLE 4. Bactericidal activities of pooled chinchilla antisera with agammaglobulinemic human sera as the complement source

Immunogen	Bactericidal activity[a] of sera tested	
	Preimmune	Immune
Whole NTHi cells	1:10	1:160
Outer membranes	1:10	1:80
Hi-PAL, rPCP, and *e* protein	1:20	1:20
PCP-PAL hybrid and *e* protein	1:10	1:10
Saline (control)	1:10	<1:10

[a] Reciprocal of the highest dilution of sera killing >50% of NTHi test strain 860295.

was just above the 50% killing end point at a 1:10 dilution (thus a titer of 10) and the immune sera was just below this point (a titer of <10). To be significant, bactericidal activity must be fourfold above the background level (i.e., two wells) and the immune sera from the saline group was not more active than the preimmune sera. This degree of variability is normal for this assay.

To determine whether chinchilla antibodies (presumably immunoglobulin G) can fix complement on the surfaces of NTHi cells, an ELISA was developed to measure C3 fixed on formalin-treated NTHi cells. This assay was sensitive and specific for guinea pig C3 fixed by antibodies. Titers of the antisera and normal sera in the C3 fixation assay are shown in Table 3. All of the immune sera show titers of fixed C3, while the preimmune sera and the saline control sera show very low background titers, indicating that the elicited antibodies are responsible for the fixed C3. The failure of the anti-Hi-PAL, rPCP, plus *e* protein and anti-rPCP-PAL hybrid plus *e* antisera to kill NTHi does not seem to be due to an inability of chinchilla antibodies to fix complement, as both of the positive-control antisera can kill the NTHi 860295 target strain and all antisera can fix detectable C3 on the surfaces of NTHi cells. In fact, the levels of complement-fixing antibodies in the protective anti-whole cell sera are considerably lower than those in the nonprotective sera directed against the purified protein mixtures. Thus, levels of surface-reactive C3-fixing antibodies do not correlate with protection from infection or disease in this animal model.

DISCUSSION

OMPs of NTHi have been investigated extensively as potential vaccine candidates for otitis media, sinusitis, and pneumonia. Many of the OMPs of NTHi are capable of eliciting biologically active antisera in in vitro assay systems. Antisera directed against some of the OMPs have been shown to be protective in models of Hib meningitis (12–14, 19, 24, 25) and antibodies against P1 have been reported to be protective in the chinchilla model of otitis media (30).

Two of the more well-studied OMPs of *H. influenzae* include the Hi-PAL (P6) protein and the *e* (P4) protein. While much is known about protective effects of anti-Hi-PAL and anti-*e* protein sera against Hib, little work has been published on the in vivo effects of these antibodies on NTHi. This laboratory has recently reported that mixtures of antisera directed against these proteins show synergistic killing of both Hib and NTHi cells in the in vitro bactericidal assay (5, 12, 14). Thus, it was decided to test these immunogens along with the rPCP protein in an animal model of NTHi infection.

None of the animal models used for NTHi infection are

wholly satisfactory. There are animal models for lung infection (16, 34) which range from enhanced pulmonary clearance to suspending organisms in agarose beads for prolonged infection. Models of otitis media are somewhat more suitable, with the chinchilla and rat being two of the more well documented. While neither of these two is completely satisfactory, we selected the chinchilla for in vivo testing of the Hi-PAL, rPCP, and *e* immunogens. Previous studies using the chinchilla have demonstrated that parenteral immunization can result in protection (30) and that passive administration of antibodies against whole cells could also protect. This protection was not due to antibodies against lipooligosaccharide (1).

Preliminary studies in mice indicated that alum was effective as an adjuvant for the proteins described in this study and allowed elicitation of biologically active antibodies (data not shown). However, chinchillas immunized with vaccines and alum had low ELISA titers. When animals were challenged with an average of 50 viable NTHi 860295 directly into the middle ear, results were disappointing in that all vaccine groups, including the positive-control group (immunized with outer membranes from the challenge strain) failed to protect the animals from infection or to influence the disease course in any way. Thus, it was decided to lower the challenge dose to approximately 10 organisms per ear, as in previously published experiments with this model (18). Since aluminum hydroxide was unsuccessful in raising protective antisera even in the positive-control group, it was discarded as an adjuvant for chinchilla studies and IFA, which was successful in published experiments (1), was used as a substitute.

Because of the reported synergy of the anti-Hi-PAL, anti-*e* protein, and anti-rPCP sera (5, 12), the purified proteins were mixed prior to immunization. It was hoped that this mixture would result in much greater biologic activity and would overcome the perceived problem of the large number of doses of purified P1 required to achieve protection previously reported by Pelton et al. (30).

The protection data reported in this report further extend the results of Barenkamp (1) by showing that immunization with outer membranes can protect chinchillas against the homologous NTHi strain. The chinchillas immunized with the mixture of the three lipoproteins did show a trend toward some modification of the symptoms of otitis with statistically significant reductions in effusion (Fig. 3) and inflammation as measured by tympanometry. However, the proteins used as experimental vaccines failed to protect the animals from infection or to significantly alter the disease course, as measured by otoscopy (Table 1 and Fig. 2). This lack of protection was surprising, given the conserved antigenic nature of the proteins, their surface exposure, and the bactericidal and, in two cases, protective antibody responses to the proteins in other animals. However, when the chinchilla antisera elicited in response to these proteins were examined, we were unable to demonstrate detectable bactericidal activity. Previous studies (5, 6, 12, 14) demonstrated that each of these proteins individually elicits bactericidal antibodies in mice and rabbits against multiple NTHi strains and failed to show any blocking activity when anti-rPCP and anti-Hi-PAL or anti-Hi-PAL and anti-*e*-protein sera were used. Two possible explanations for the failure of these vaccines to protect in the chinchilla model are as follows. (i) The proteins in the vaccine preparations denatured during purification or were otherwise altered in conformation. (ii) The immune responses of chinchillas to these immunogens were different.

Prior to mixing, the purified proteins used as immunogens were probably in conformations which elicit biologically active antibodies, since a rabbit antiserum produced with a similar lot of Hi-PAL was tested in the infant rat assay and found to be protective against Hib (data not shown). Also, we have never had a Hi-PAL or *e*-protein preparation fail to elicit bactericidal antibodies in rabbits. However, these chinchilla experiments represent the first time that these purified proteins have been mixed prior to immunization. Since the calculated pI of Hi-PAL is acidic and the pI of *e* protein is basic, it is conceivable that these proteins could have formed a complex, which might result in an altered conformation. Similar possibilities exist for the rPCP-PAL hybrid protein and *e* protein. Experiments to test this possibility are planned.

Another explanation of these results is that the immune responses of chinchillas to these proteins may vary from that of previously tested animals and humans. The chinchilla antibodies which were elicited can fix complement, as demonstrated by the presence of C3 fixed on the surfaces of whole NTHi cells in the complement fixation ELISA assay (Table 2). Thus, there does not seem to be a problem with the ability of chinchilla immunoglobulin G and/or immunoglobulin M to initiate the complement cascade. Since blocking antibodies directed against particular epitopes of these proteins have never been demonstrated in any other animal species, a more likely explanation is that the chinchillas do not produce antibodies against the bactericidal epitopes of the proteins. It is unknown whether bactericidal antibodies have any role in protection in the chinchilla model, but there does seem to be a correlation between their presence and protection from infection. In humans, bactericidal antibodies do not seem to have any effect on colonization of the human nasopharynx (2) but do seem to have an effect on protection from otitis due to NTHi (9), so the absence of bactericidal antibodies could be responsible for the failure of the purified proteins to protect these animals from experimental otitis media. If this is the case, then the chinchilla may not be an appropriate model to test the vaccine potentials of these OMPs or possibly other OMPs, using active immunization.

While the possibility remains that these proteins are not protective antigens against otitis media caused by NTHi, the significant reduction in inflammation and effusion provided by the mixture of Hi-PAL, rPCP, and *e* protein does indicate some promise for these vaccine candidates. Additional experiments to clarify these points and to further evaluate the value of these proteins and combinations thereof for vaccines against NTHi otitis media will be needed.

ACKNOWLEDGMENTS

We thank Stacey Meredith for help in developing the complement fixation ELISA, Nancy Strong and Nan Silliman for help with ELISAs, and Jan Stephenson for microbiological assistance.

A portion of this work was supported by grant R01 AI 25233-03 (to B.A.G.) from the National Institute of Allergy and Infectious Diseases.

REFERENCES

1. **Barenkamp, S. J.** 1986. Protection by serum antibodies in experimental nontypable *Haemophilus influenzae* otitis media. Infect. Immun. **52**:572–578.
2. **Bernstein, J. M., H. S. Faden, and P. L. Ogra.** 1991. Nasopharyngeal colonization by nontypeable *Haemophilus influenzae* in children: the effect of serum bactericidal antibody. Otolaryngol. Head Neck Surg. **105**:406–410.
3. **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. W. B. Saunders Co., Philadelphia.

4. **Brinton, C. C., Jr., M. J. Carter, D. B. Derber, S. Kar, J. A. Kramarik, A. C. C. To, and S. W. Wood.** 1988. Design and development of pilus vaccines for *Haemophilus influenzae* diseases. Pediatr. Infect. Dis. J. **8**:54–61.
5. **Deich, R. A., A. Anilionis, J. Fulginiti, B. J. Metcalf, S. Quataert, T. Quinn-Dey, G. W. Zlotnick, and B. A. Green.** 1990. Antigenic conservation of the 15,000-dalton outer membrane lipoprotein PCP of *Haemophilus influenzae* and biologic activity of anti-PCP antisera. Infect. Immun. **58**:3388–3393.
6. **Deich, R. A., A. Anilionis, J. P. Fulginiti, T. Quinn-Dey, G. W. Zlotnick, and B. A. Green.** 1992. Recombinant hybrids of *Haemophilus influenzae* (Hi) outer membrane lipoproteins as vaccine candidates., abstr. E69, p. 155. Abstr. Gen. Meet. Am. Soc. Microbiol. 1992. American Society for Microbiology, Washington, D.C.
7. **DeMaria, T. F., B. R. Briggs, D. J. Lim, and N. Okazaki.** 1984. Experimental otitis media with effusion following middle ear inoculation of nonviable *H. influenzae*. Ann. Otol. Rhinol. Laryngol. **93**:52–56.
8. **Doyle, W. J., J. S. Suppance, G. Marshak, E. Y. Cantekin, C. D. Bluestone, and D. D. Rohn.** 1982. An animal model of acute otitis media consequent to β lactamase producing non-typable *Haemophilus influenzae*. Otolaryngol. Head Neck Surg. **90**:831–836.
9. **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 nontypable *Haemophilus influenzae*. J. Infect. Dis. **160**:999–1004.
10. **Giebink, G. S., E. E. Payne, E. L. Mills, S. K. Juhn, and P. G. Quie.** 1976. Experimental otitis media due to *Streptococcus pneumoniae*: immunopathologic response in the chinchilla. J. Infect. Dis. **134**:595–604.
11. **Giebink, G. S., M. L. Ripley-Petzoldt, S. K. Juhn, D. Aeppli, A. Tomasz, and E. Tuomanen.** 1988. Contribution of pneumococcal cell wall to experimental otitis media pathogenesis. Ann. Otol. Rhinol. Laryngol. **97**:28–30.
12. **Green, B. A., J. E. Farley, T. Quinn-Dey, R. A. Deich, and G. W. Zlotnick.** 1991. The *e* (P4) outer membrane protein of *Haemophilus influenzae*: biologic activity of anti-*e* serum and cloning and sequencing of the structural gene. Infect. Immun. **59**:3191–3198.
13. **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.
14. **Green, B. A., T. Quinn-Dey, and G. W. Zlotnick.** 1987. Biologic activities of antibody to a peptidoglycan-associated lipoprotein of *Haemophilus influenzae* against multiple clinical isolates of *H. influenzae* type b. Infect. Immun. **55**:2878–2883.
15. **Haase, E. M., A. A. Campagnari, J. Sarwar, M. Shero, M. Wirth, C. U. Cumming, and T. Murphy.** 1991. Strain-specific and immunodominant surface epitopes of the P2 porin protein of nontypeable *Haemophilus influenzae*. Infect. Immun. **59**:1278–1284.
16. **Hansen, E. J., D. A. Hart, J. L. McGehee, and G. B. Toews.** 1988. Immune enhancement of pulmonary clearance of nontypable *Haemophilus influenzae*. Infect. Immun. **56**:182–190.
17. **Hermansson, A., P. Emgård, K. Prellner, and S. Hellström.** 1988. A rat model for pneumococcal otitis media. Am. J. Otolaryngol. **9**:97–101.
18. **Karasic, R., D. J. Beste, S. C.-M. To, W. J. Doyle, S. J. Wood, M. J. Carter, A. C. C. To, K. Tanpowpong, C. D. Bluestone, and C. C. Brinton, Jr.** 1988. Evaluation of pilus vaccines for prevention of experimental otitis media caused by nontypable *Haemophilus influenzae*. Pediatr. Infect. Dis. J. **8**(Suppl.):S62–S65.
19. **Kimura, A., P. A. Gulig, G. H. McCracken, Jr., T. A. Loftus, and E. J. Hansen.** 1985. A minor high-molecular-weight outer membrane protein of *Haemophilus influenzae* type b is a protective antigen. Infect. Immun. **47**:253–259.
20. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
21. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
22. **Miller, J. H.** 1972. Experiments in molecular genetics, p. 203. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
23. **Mills, R., and J. Gilsdorf.** 1986. Middle-ear effusions following acute otitis media in the chinchilla animal model. J. Laryngol. Otol. **100**:255–261.
24. **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.
25. **Munson, R. S., Jr., J. L. Shenep, S. J. Barenkamp, and D. M. Granoff.** 1983. Purification and comparison of outer membrane protein P2 from *Haemophilus influenzae* type b isolates. J. Clin. Invest. **72**:677–684.
26. **Murphy, T. F., L. C. Bartos, A. M. Campagnari, M. B. Nelson, K. C. Dudas, and M. A. Apicella.** 1986. Antigenic characterization of the P6 protein of nontypable *Haemophilus influenzae*. Infect. Immun. **54**:774–777.
27. **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 bactericidal antibody. J. Clin. Invest. **78**:1020–1027.
28. **Murphy, T. F., M. B. Nelson, K. C. Dudas, J. M. Mylotti, and M. A. Apicella.** 1986. Identification of a specific epitope of *Haemophilus influenzae* on a 16,600 dalton outer membrane protein. J. Infect. Dis. **152**:1300–1307.
29. **Musher, D. M., K. R. Kubitshek, J. Crennan, and R. E. Baughn.** 1983. Pneumonia and acute febrile tracheobronchitis due to *Haemophilus influenzae*. Ann. Intern. Med. **99**:344–350.
30. **Pelton, S. I., G. Bolduc, S. Guiati, Y. Liu, and P. A. Rice.** 1990. Protection from experimental otitis media in chinchillas due to nontypable *Haemophilus influenzae* following immunization with outer membrane protein 1 (P1), abstr. 610, p. 188. Program Abstr. 30th Intersci. Conf. Antimicrob. Agents Chemother. 1990. American Society for Microbiology, Washington, D.C.
31. **Peterson, G. L.** 1977. A simplification of the protein method of Lowry *et al.* which is more generally applicable. Anal. Biochem. **83**:346–356.
32. **Reilly, J. S., W. J. Doyle, E. I. Cantekin, J. S. Supance, C. K. Ha-Kyung, D. D. Rohn, and C. D. Bluestone.** 1983. Treatment of ampicillin-resistant acute otitis media in the chinchilla. Arch. Otolaryngol. **109**:533–535.
33. **Shurin, P. A., G. S. Giebink, D. L. Wegman, D. Ambrosino, J. Rholl, M. Overman, T. Bauer, and G. R. Siber.** 1988. Prevention of pneumococcal otitis media in chinchillas with human bacterial polysaccharide immune globulin. J. Clin. Microbiol. **26**:755–759.
34. **Slater, L. N.** 1990. A rat model of prolonged pulmonary infection due to nontypable *Haemophilus influenzae*. Am. Rev. Respir. Dis. **142**:1429–1435.
35. **Tsai, C. M., and C. E. Frasch.** 1983. A sensitive stain for detecting lipopolysaccharide in polyacrylamide gels. Anal. Biochem. **119**:115–119.
36. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33**:103–119.
37. **Zlotnick, G. W., V. T. Sanfillippo, 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.