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The Acylated Form of Protein D of *Haemophilus influenzae* Is More Immunogenic than the Nonacylated Form and Elicits an Adjuvant Effect When It Is Used as a Carrier Conjugated to Polyribosyl Ribitol Phosphate

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The nonacylated form of protein D (PDm) of *Haemophilus influenzae* has been shown to induce the production of antibodies that are bactericidal to homologous and heterologous nontypeable *H. influenzae* (NTHi) strains. In this study, immunization of rats with lipoprotein D (LPD) induced higher levels of anti-protein D immunoglobulin G and A serum antibodies than immunization with PDm, and the bactericidal activities of sera from LPD-immunized rats were greater than those of sera from PDm-immunized rats. Immunization with LPD or PDm did not prevent the development of acute otitis media (AOM) when rats were challenged with 10^4 CFU of an NTHi strain. However, on the eighth day of bacterial challenge, 50% (5 of 10) of LPD-immunized rats had recovered from otitis media and 30% (3 of 10) had negative middle ear cultures, whereas only 30% (3 of 10) of PDm-immunized rats had recovered, though none was culture positive. Immunization with an inactivated homologous bacterial strain elicited 70% protection (i.e., 7 of 10 rats) in the rat otitis media model. LPD and PDm were also conjugated to the *H. influenzae* type b (Hib) capsular polysaccharide, polyribosyl ribitol phosphate (PRP), to test protein D-conjugated PRP vaccine's potential for protection against Hib infection. When two LPD-conjugated and two PDm-conjugated PRP vaccines, each containing a different protein concentration, and a tetanus toxoid-conjugated vaccine (ACT-HIB) were tested in the experimental model of rat otitis induced with a Hib strain (Minn A), both of the LPD-conjugated and one of the PDm-conjugated vaccines induced significant protection from AOM, the level of protection being highest in animals given the vaccine with the highest LPD content. Sera from these rats also manifested the highest anti-PRP and anti-LPD antibody levels and the highest bactericidal activities against a Hib strain and an NTHi strain.

The *Haemophilus influenzae* capsular polysaccharide, polyribosyl ribitol phosphate (PRP), plays a major role in determining the types of infections caused by this organism. Unencapsulated *H. influenzae* strains are responsible primarily for mucosal infections, such as otitis media, sinusitis, pneumonia, and pharyngitis (12, 31, 35), whereas encapsulated strains cause more invasive diseases, such as meningitis, epiglottitis, suppurative arthritis, cellulitis, and otitis media (8, 28). Currently available *H. influenzae* type b (Hib) vaccines have been shown to be effective only against infections caused by Hib. These vaccines consist of PRP coupled to a protein carrier, and they induce protectively high levels of anti-PRP antibodies even in infants as young as 2 to 3 months old (9, 16). None of the currently available Hib vaccines use *H. influenzae* proteins as carriers (9); thus, they are not effective against nontypeable *H. influenzae* (NTHi). Natural immunity to NTHi starts to develop at 2 years of age, as shown by seroconversion and the presence of serum antibodies specific to outer membrane proteins (OMPs) of *H. influenzae* (3, 36). The occurrence and biological activity of serum antibodies to OMPs of NTHi have been shown to be associated with protection from NTHi-induced otitis media (6, 32). This observation has led to the testing of several OMPs of *H. influenzae* as vaccines against *H. influenzae* infection (6, 10, 18, 20, 29, 30).

In earlier studies by members of our laboratory, a nonacylated form (PDm) of a 42-kDa membrane lipoprotein (protein D) that is conserved in both NTHi and encapsulated *H. influenzae* strains was purified and shown to induce bactericidal antibodies against homologous and heterologous NTHi strains (1, 3). Measurement of serum anti-PDm antibody levels in human donors of different ages showed that the levels of immunoglobulin G (IgG) antibodies start to increase after 1 year of age and that those of IgA antibodies start to increase after 2 years, whereas IgM antibody levels start to increase as early as 6 months of age (3). Protein D was among the *H. influenzae* membrane proteins found to be targets of serum antibodies of rats recovering from experimentally induced acute otitis media (AOM) (27). Furthermore, rats immunized intranasally or perorally with NTHi developed saliva IgA antibodies to protein D (2). In a study by other investigators, lipoprotein D (LPD) was shown to potentiate antibody responses to T-cell-independent type 2 antigens (bacterial polysaccharides) in an in vitro culture system (33).

In the present study, the antigenicities of native LPD and PDm were compared for groups of rats immunized with these forms of protein D. The biological activities of the rat sera were tested against NTHi and Hib strains in a bactericidal assay, and the potentials for protection with LPD and PDm immunization were compared in a rat model of NTHi-induced AOM. The studies were extended to test the carrier effects of

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LPD and PDm on the immunogenicities and potentials for protection of PRP conjugate vaccines by immunizing rats with vaccines synthesized by coupling PRP to LPD or PDm.

MATERIALS AND METHODS

Bacterial strains. Hib strain Minn A and NTHi strain 3655 were kind gifts from R. Munson, St. Louis, Mo. NTHi strain 772 was an isolate from a patient at University Hospital, Malmö, Sweden. All strains were grown overnight at 37°C with aeration in 1 liter of brain heart infusion broth (Difco Laboratories) containing hemin (10 μg/ml) (Sigma Chemical Co., St. Louis, Mo.) and NAD (10 μg/ml) (Sigma) or were grown on chocolate agar plates by incubation overnight at 37°C in an atmosphere containing 5% CO_2.

PDm and LPD. PDm from an *Escherichia coli* strain overexpressing PDm [*E. coli* (pHIM502)] in its periplasmic space was purified as described previously (3). LPD, obtained from SmithKline Beecham Biologicals (Rixensart, Belgium), is a recombinant protein expressed in *E. coli* AR58 following amplification of the gene from NTHi strain 772 by PCR and cloning in a pMC27N-derived plasmid. pMC27N is a pBR 322 derivative in which the expression of a heterologous gene is under the control of a pL promoter. LPD was extracted and purified with conventional chromatographic sorbents. The purities of LPD and PDm were assessed by scanning of Coomassie-stained sodium dodecyl sulfate (SDS)-polyacrylamide gels with a laser-based densitometer (Personal Densitometer SI; Molecular Dynamics, Sunnyvale, Calif.).

PRP vaccines. A virulent strain of Hib was grown in a synthetic medium supplemented with hematin in a fermentor under regulated conditions. The culture was treated with Cetavlon to precipitate the PRP, which was recovered by filtration. Thereafter, PRP was selectively dissolved in water-ethanol diluent and further purified by selective precipitation. Native capsular polysaccharide was used for conjugation to the carrier protein. The product met the World Health Organization requirements for Hib conjugate vaccines with regard to moisture, ribose, phosphorus, protein, nucleic acid, and endotoxin content and consistency of molecular size.

PRP was directly conjugated to LPD and PDm following activation of PRP with organic cyanylated agent. The conjugate was purified by gel filtration chromatography on a Sephacryl HR500 column in 0.2 M NaCl. The PRP content in the conjugate preparations was measured by orcinol reaction with D-ribose as the standard (4), and the protein content was determined by the Lowry method. LPD and PDm vaccines were each at two different concentrations. The first LPD vaccine (PRP-LPD-1) contained 72 μg of PRP per ml conjugated to 88 μg of protein carrier per ml, and the second (PRP-LPD-2) contained 99 μg of PRP per ml and 60 μg of protein per ml. The corresponding concentrations for the two PDm vaccines were 114 μg of PRP per ml plus 110 μg of protein per ml for the first vaccine (PRP-PDm-1) and 29.4 μg of PRP per ml plus 70 μg of protein per ml for the second vaccine (PRP-PDm-2). ACT-HIB (Pasteur Merieux, Lyon, France) vaccine contains 20 μg of PRP per ml conjugated to 48 μg of tetanus toxoid per ml (PRP-T).

Immunization of rats. For challenge with 10^4 CFU of NTHi strain 3655, four groups, each containing 10 rats, were immunized. Groups I and II were subcutaneously immunized with 100 μg of LPD and 100 μg of PDm, respectively, which were injected in 100-μl volumes. A negative control group (group III) received 100 μl of phosphate-buffered saline (PBS) and a positive control group (group IV) received 200 μl of formaldehyde-inactivated NTHi strain 3655. To inactivate NTHi strain 3655, log-phase-grown bacteria were suspended in 2% formaldehyde at a concentration of 10^9 CFU/ml and left on ice for 30 min. Bacteria were washed three times, and rats were immunized with 10^6 CFU of bacteria. In a separate study, groups of rats immunized with LPD or injected with PBS were challenged with 50 CFU of NTHi strain 3655. Twelve rats were immunized subcutaneously with 100 μg of LPD in a 100-μl volume, and the negative control group (12 rats) was injected with PBS. All vaccines were prepared by mixing them with equal amounts of complete Freund's adjuvant. All groups were given boosters twice with the same amount of antigen 3 and 5 weeks after the initial dose. LPD and PDm were mixed with incomplete Freund's adjuvant for booster immunization. Blood samples were collected from tail veins or by cardiac puncture prior to the first immunization and on the day of bacterial challenge.

Protein D-coupled PRP vaccines were tested for protection against Hib-induced otitis media. Vaccines were subcutaneously introduced into six groups of 12-week-old Sprague-Dawley rats weighing 200 g as follows: groups A and B, PRP-LPD-1 and PRP-LPD-2 vaccines, respectively; groups C and D, PRP-PDm-1 and PRP-PDm-2 vaccines, respectively; group E, PRP-T vaccine (ACT-HIB); and group F, sham (PBS) vaccine. Each group contained six rats. For groups A to E, the PRP content of each vaccination was adjusted to 5 μg by varying the volumes administered. Booster doses of the same strength were given 3 weeks later. To study the bactericidal activities of antibodies to PDm and LPD against Hib strain Minn A, two rats were subcutaneously immunized and given boosters as described above. Lastly, one rat was intraperitoneally immunized with inactivated NTHi strain 3655, also as described above. Immune sera were collected 10 days after the final immunization. Blood was drawn from each rat 10 days after the last immunization, and sera were tested with a bactericidal assay against Hib strain Minn A.

Induction of experimental otitis media. Challenge of rats with NTHi strain 3655 and Hib strain Minn A was performed exactly as described previously (2). In the first NTHi infection study, 10 days after the second booster vaccination dose 18-week-old Sprague-Dawley rats, weighing 300 to 350 g, were challenged with 10^4 CFU of NTHi strain 3655 grown to log phase. In the second study, where a group of LPD-immunized rats and a group of PBS-inoculated rats were included, rats were challenged with 50 CFU of NTHi strain 3655. Rats that were challenged with Hib strain Minn A were also given 50 CFU of bacteria grown to log phase. Bacteria were injected into the middle-ear cavity in 50-μl volumes after the bulla had been exposed through a ventral incision in the neck. The ears of the rats were examined otoscopically 4 and 8 days after bacterial challenge, diagnosis of AOM being based on the presence of purulent effusion, and tympanic vascular changes were observed. After the last otoscopy examination, middle ear culture specimens were taken with a sterile cotton-tip applicator through the pierced tympanic membrane.

SDS-PAGE and Western blot assay. Preparation of sonication extracts of NTHi strain 3655 and NTHi strain 772 from broth-grown bacteria and the running of SDS-polyacrylamide gel electrophoresis (PAGE) were done exactly as described before (1, 3). Boiled samples were added to SDS-PAGE gels in buffers containing 1% (wt/vol) β-mercaptoethanol. Staining of gels was done with Coomassie brilliant blue G-250 (Sigma) according to the manufacturer's instructions. Protein bands were also transferred to nitrocellulose membranes (Sartorius, Göttingen, Germany) exactly as described previously (1, 3). Pooled immune sera from rats belonging to different groups were incubated overnight with membrane strips at 1:200 dilutions. Peroxidase-labelled secondary antibodies were goat anti-rat immunoglobulins (Sigma) and were added at 1:2,000 dilutions.

ELISA. Rat sera were examined for anti-protein D antibodies with an enzyme-linked immunosorbent assay (ELISA) as described previously (2, 3). Levels of serum IgG and IgA antibodies directed to LPD, PDm, and PRP were measured in the immunized rats. Both of the protein D antigens (LPD and PDm) were used to coat flat-bottomed gamma-irradiated polystyrene microtiter plates (Immunoplate I; Nunc A/S, Tåstrup, Denmark) at concentrations of 50 ng/well by incubating the plates at 4°C overnight in 100-μl volumes of 0.1 M $NaHCO_3$ buffer (pH 9.6). Diphtheria toxoid-conjugated PRP vaccine (ProHIBIT; Connaught Laboratories, Swiftwater, Pa.) was added at a PRP concentration of 50 ng/well to measure anti-PRP antibody levels. A lane of goat anti-rat IgG (50 ng/well; Sigma Immunochemicals) or goat anti-rat IgA (Nordic, Capistrano Beach, Calif.) was included in each plate to calculate the relative concentrations of antibodies directed against 50 ng of LPD, PDm, or PRP. Following the blocking of wells with blocking buffer (0.05% Tween 20 in 2% bovine serum albumin [BSA]–PBS) for 2 h, the plates were washed with 0.05% Tween 20–PBS (washing buffer), and rat sera serially diluted in blocking buffer were added to wells containing LPD, PDm, or PRP-diphtheria toxoid. To wells containing goat anti-rat IgG or goat anti-rat IgA were added serially diluted predetermined concentrations of purified rat IgG (Sigma Immunochemicals) or purified rat IgA (The Binding Site, Birmingham, Great Britain). After 2 h of incubation with the sera, peroxidase-conjugated secondary antibodies, goat anti-rat whole-molecule IgG (Sigma Immunochemicals) or IgA α-chain (Nordic), were added to the wells at a 1:2,000 dilution. To facilitate color development, 20 mM tetramethylbenzidine, pH 4.25, with 0.004% hydrogen peroxide (final concentration) was used as the substrate for peroxidase. Before the optical density (OD) at 450 nm was measured with an automated microtiter plate reader (Organon Teknika, Turnhout, Belgium), the enzymatic reaction was stopped by adding 1 M sulfuric acid. The relative amounts of antibodies directed to 50 ng of antigen were calculated by comparing the OD values of the rat sera with those of a standard curve obtained with specific concentrations of purified rat IgG and IgA. Because the predetermined concentrations of IgG and IgA were always added to each plate from the same stock solutions, plate-to-plate variation could be calculated with the OD values for the standard. This method allowed the measurement of antibody levels as low as approximately 5 ng/ml when 50 ng of antigen was plated.

Bactericidal assay. The levels of bactericidal activity of immune sera were determined as described previously (3). Sera were incubated at 56°C for 30 min to remove complement activity. NTHi strain 3655 or Hib strain Minn A was grown to log phase in brain heart infusion broth and suspended at 5×10^4 CFU/ml in sterile PCM buffer (PBS with 0.15 mM $CaCl_2$ and 1 mM $MgCl_2$) containing 1% BSA. Sera from four-day-old infant rats were used as the complement source, and aliquots of pooled sera were kept at −70°C. The reagents were added to round-bottomed polystyrene Falcon tubes (Becton Dickinson, Paramus, N.J.) in the following order: 20 μl of bacteria, 20 μl of serially diluted antiserum, 20 μl of complement, and finally 40 μl of 1% BSA in PCM buffer. To determine the initial bacterial concentration, 10-μl aliquots of the mixture were plated on chocolate agar plates at time zero. Following incubation of the tubes at 37°C for 1 h with shaking, 10 μl of the sample was plated in duplicate from each tube. A tube with the serum sample omitted was included in each experiment to ensure that the complement itself did not manifest killing, and a tube containing the test serum but no complement was included to ensure that serum complement was successfully inactivated. The agar plates were left at 37°C overnight, and the bactericidal activities of serially diluted antisera were assessed by determining the numbers of CFU the following day. The dilutions manifesting 50% killing were noted for comparison of the activities of different sera. Experiments were repeated at least three times.

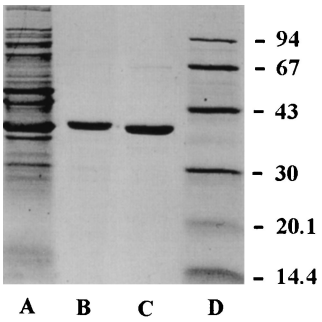

FIG. 1. Results of SDS-PAGE of Coomassie-stained NTHi strain 772 sonication extract (lane A), purified LPD (lane B), purified PDm (lane C), and molecular mass marker (lane D). Molecular masses are in kilodaltons. Ten micrograms of material was added to each well.

Statistics. The statistical significance of differences in the bactericidal activities of antisera was assessed with the Mann-Whitney U test. Student's *t* test was used to compare the mean ELISA values, and Fisher's exact test was used to compare the AOM assessments for the sham (PBS)-immunized group with those for the rest of the groups. In all assays, a P value of <0.05 was considered significant.

RESULTS

Assessment of the purity of protein D. The purities of LPD and PDm were assessed by SDS-PAGE and Western blot assay. Coomassie staining of the gel demonstrated a single band at around 42 kDa on LPD- and PDm-treated wells (Fig. 1). Scanning of the stained gel with a laser-based densitometer revealed more than 95% purity for both of the protein D preparations.

Serum anti-protein D and anti-PRP antibody levels. Blotting of pooled immune sera from rats belonging to different groups that were subsequently challenged with NTHi strain 3655 against an NTHi strain 3655 OMP preparation demonstrated that antibodies from rats immunized with LPD and PDm detected a single 42-kDa band corresponding to protein D (Fig. 2). In the same assay, pooled sera from NTHi strain 3655-immunized rats detected all the major OMPs. Individual immune sera from the same group of animals immunized with LPD and PDm were tested to determine levels of anti-LPD IgG and IgA antibodies (Fig. 3). Mean values for each group were noted for statistical comparison. Animals immunized with LPD had higher anti-LPD IgG and IgA concentrations (107.7 ± 23.25035 μg/ml [mean ± standard error] and 10.5 ± 1.16252 μg/ml, respectively) than rats immunized with PDm (IgG, 43.4 ± 9.97465 μg/ml; IgA, 4.5 ± 0.8587 μg/ml), and the difference between LPD- and PDm-immunized rats was statistically significant ($P < 0.05$). Immunization of rats with NTHi strain 3655 yielded significantly lower concentrations of IgG (0.616 μg/ml) and IgA (0.104 μg/ml) antibodies against LPD. The mean IgG and IgA antibody levels of the LPD-immunized group which was later challenged with 50 CFU of NTHi strain 3655 were comparable to those of the above-mentioned group of rats immunized with LPD prior to challenge with 10^4 CFU of NTHi strain 3655.

Ten days after the booster dose, immune sera from the rats immunized with the protein D-conjugated PRP vaccine were tested for antibody responses to PRP, LPD, and PDm by ELISA. Immunoblotting of these sera against an NTHi strain 3655 OMP preparation and of the sera from LPD- and PDm-immunized rats against a Hib strain Minn A OMP preparation by Western blot assay revealed a single 42-kDa protein corresponding to protein D (data not shown). Sera from rats immunized with one of four protein D vaccines or the PRP-T vaccine had high levels of anti-PRP IgG and IgA antibodies (Fig. 4). The highest mean anti-PRP IgG antibody levels

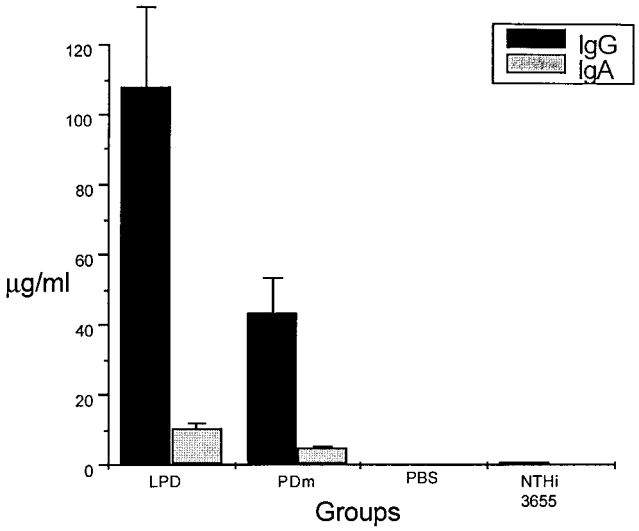

FIG. 3. Levels of anti-LPD IgG and IgA antibodies in sera of rats treated with LPD (group I), PDm (group II), PBS (group III), or NTHi strain 3655 (group IV).

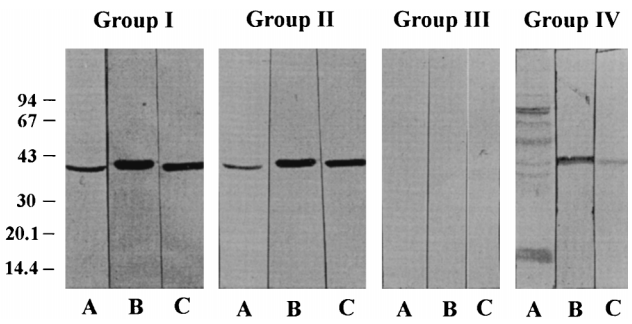

FIG. 2. Western blot analysis of pooled rat sera from groups I to IV against sonication extract of NTHi strain 3655 (lanes A), purified LPD (lanes B), or purified PDm (lanes C). Molecular masses are in kilodaltons.

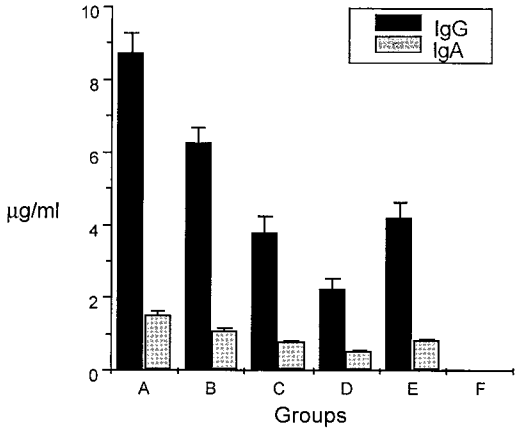

FIG. 4. Serum anti-PRP antibody levels in rats immunized with PRP-LPD (groups A and B), PRP-PDm (groups C and D), or PRP-T (group E) or injected with PBS sham vaccine (group F).

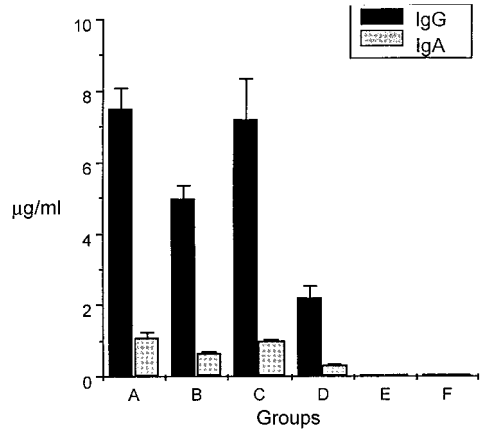

FIG. 5. Serum anti-LPD antibody levels in rats immunized with PRP-LPD (groups A and B), PRP-PDm (groups C and D), or PRP-T (group E) or injected with PBS sham vaccine (group F).

(8.72 ± 0.5512 μg/ml and 6.24 ± 0.41534 μg/ml) occurred in groups A and B, which were immunized with LPD-containing conjugate vaccines. The vaccine used in group A contained twice the amount of LPD present in the group B vaccine. Sera from PRP-T-immunized rats (group E) manifested the third-highest IgG levels. Mean anti-PRP IgG antibody levels were significantly higher in group A than in any other group ($P < 0.05$). Group A also manifested the highest anti-PRP IgA antibody levels, followed by groups B, E, C, and D, in that order (Fig. 4). Both LPD- and PDm-conjugated vaccines induced the production of IgG and IgA antibodies to LPD (Fig. 5) and PDm (data not shown). The concentrations of LPD and PDm in the conjugate vaccines also influenced the development of IgG and IgA antibodies against protein components. Group A exhibited the highest level of anti-LPD antibodies, and group C exhibited the highest level of anti-PDm antibodies.

Serum bactericidal activity of LPD- and PDm-immunized rats and assessment of AOM. Immune sera of the animals subsequently challenged with NTHi strain 3655 were pooled groupwise and tested against NTHi strain 3655 in a bactericidal assay. The mean values of serum dilutions yielding 50% killing in three separate experiments were noted for comparison. Pooled sera from group IV manifested the highest levels of bactericidal activity, with 50% killing at a dilution of 1:42 ($P < 0.05$). Sera of LPD-immunized rats (group I) manifested 50% killing at a dilution of 1:6 ($P < 0.05$), compared with a dilution of 1:3 ($P < 0.05$) for the sera of PDm-immunized rats (group II). Sera of sham-immunized rats (group III) manifested no bactericidal activity.

In the first study of AOM, where rats were challenged with 10^4 CFU of NTHi strain 3655, otoscopy performed 4 days after bacterial challenge showed 7 of 10 rats ($P < 0.05$) in group IV (i.e., immunized with NTHi strain 3655) to be free from middle ear pus (Table 1). All animals in the other three groups had pus in their ears. The second otoscopy examination, performed on the eighth day of bacterial challenge, showed all the rats in the NTHi-immunized group to be pus free ($P < 0.05$) and that clearing of middle ear pus had started in the other groups as well. On this day, 50% of the rats (5 of 10) in group I (i.e., immunized with LPD) had recovered ($P > 0.05$), whereas in group II (immunized with PDm) 30% of the rats (3 of 10) ($P > 0.05$) were without middle ear pus. Two of the rats (20%) in group III (controls) were considered to have recovered on the eighth day. Bacterial cultures of specimens collected from ears on the eighth day showed none of the rats in group IV to be positive, whereas 7 of 10 of group I (70%), 10 of 10 of group II (100%), and 8 of 10 of group III (80%) rats were culture positive for NTHi. In the second study, where rats were challenged with a smaller inoculum (50 CFU) of NTHi strain 3655, five of the PBS-injected rats and one of the LPD-immunized rats died during cardiac puncture while blood was being drawn (Table 2). Otoscopy performed on the fourth day showed rats in both the LPD-immunized and the PBS-injected groups to have developed pus-positive otitis and vascular changes. Otoscopy performed on the eighth day showed that 5 of 11 LPD-immunized rats (45%) were pus free, whereas only 1 of 7 PBS-injected rats (14%) was pus free. Culturing of middle ear specimens taken on the eighth day showed 7 of 11 LPD-immunized rats (64%) to be negative for NTHi, whereas 3 of 7 PBS-injected rats (43%) were culture negative.

TABLE 1. Effect of systemic immunization on development of pus-positive and culture-positive otitis[a]

Group no. (treatment)	No. of animals with:		
	Presence of pus		Positive culture, day 8
	Day 4	Day 8	
I (LPD)	10	5	7
II (PDm)	10	7	10
III (PBS)	10	8	8
IV (NTHi strain 3655)	3	0	0

[a] Each group contained 10 animals. Groups were challenged with 10^4 CFU of NTHi strain 3655.

Bactericidal activities of sera from PRP-LPD- and PRP-PDm-immunized rats and assessment of AOM. Serum pools from groups A to F, which were immunized with PRP conjugate vaccines or sham immunized, and sera from two rats immunized with LPD and PDm were tested in a bactericidal assay against Hib strain Minn A. As indicated by the 50%-killing dilutions shown in Table 3, pooled sera from the PRP-LPD-immunized groups manifested the highest levels of bactericidal activity, the mean 50%-killing dilutions being 1:100 for group A and 1:74 for group B. Pooled sera from the PRP-T-immunized group (group E) manifested the third-highest level of bactericidal activity, at a 50%-killing dilution of 1:56, followed by the PRP-PDm-immunized groups, at 50%-killing dilutions of 1:32 for group C and 1:20 for group D. Groups A to E all manifested significantly higher levels of bactericidal activity than sham (PBS)-immunized group F, at a 50%-killing dilution of 1:1 ($P < 0.05$). Anti-LPD and anti-PDm sera elicited bactericidal activity at a 1:1 dilution against Hib strain Minn A, a result similar to that obtained with PBS-injected rat

TABLE 2. Effect of systemic immunization with LPD and PBS on development of pus-positive and culture-positive otitis[a]

Group no. (treatment)	No. of animals with:		
	Presence of pus		Positive culture, day 8
	Day 4	Day 8	
I (LPD)[b]	11	6	4
III (PBS)[c]	7	6	4

[a] Animals were challenged with 50 CFU of NTHi strain 3655.
[b] Eleven animals total. One of the 12 rats immunized died during cardiac puncture.
[c] Seven animals total. Five of the 12 rats immunized died during cardiac puncture.

TABLE 3. Bactericidal activities of groupwise-pooled rat sera and individual rat antiserum and protection against experimental otitis media induced by infection with Hib strain Minn A

Group	Treatment	50%-killing dilution against Hib strain Minn A	% (no.) of rats protected against Hib strain Minn A[a]	50%-killing dilution against NTHi strain 3655
A	PRP-LPD-1	1:100	83 (5)	1:8
B	PRP-LPD-2	1:74	67 (4)	NT[b]
C	PRP-PDm-1	1:32	50 (3)	1:2
D	PRP-PDm-2	1:20	33 (2)	NT
E	PRP-T	1:56	33 (2)	NT
F	PBS	1:1	0 (0)	NK[c]
	Anti-LPD	1:1	NT	NT
	Anti-PDm	1:1	NT	NT
	Anti-NTHi strain 3655	NT	NT	1:54

[a] All groups contained six rats.
[b] NT, not tested.
[c] NK, no killing.

sera (Table 3). Bactericidal activities of sera from groups A, C, and F and of anti-NTHi strain 3655 sera were also tested against NTHi strain 3655. Group A (PRP-LPD vaccine) exhibited a 50%-killing dilution of 1:8, while the 50%-killing dilution for group C (PRP-PDm vaccine) was 1:2 ($P < 0.05$). Bactericidal activity was not observed in the group injected with PBS, and the positive control serum (anti-NTHi strain 3655) manifested a 50%-killing dilution of 1:54 (Table 3).

The proportions of rats in each group that were protected from developing Hib-induced otitis media following immunization with PRP conjugate vaccines are listed in Table 3. All six of the PBS-injected rats developed AOM. Group A was the only group which manifested significant protection rate (five of six rats) compared with the sham (PBS)-immunized group (zero of six rats) ($P < 0.05$).

DISCUSSION

Our results showed that immunization of rats with LPD yielded significantly higher levels of IgG and IgA antibodies to LPD than immunization with PDm did. The impact of the lipid moiety on the immunogenicities of lipoproteins other than protein D has been studied by others. A nonlipoprotein form of *Borrelia burgdorferi* OspA was found to be less immunogenic than its native form (11), whereas a recombinant nonfatty acylated form of P6 of *H. influenzae* was found to yield antibodies that were as active in a bactericidal assay as those yielded by the native form (19).

In an earlier study workers from our laboratory showed that PDm yielded serum antibodies that were bactericidal against both homologous and heterologous NTHi strains (3). The present study demonstrated not only that LPD-immunized rats developed higher levels of serum antibodies to LPD than did PDm-immunized rats but also that their sera manifested greater bactericidal activity against NTHi strain 3655.

The chinchilla and rat are two species commonly used to study NTHi-induced otitis media (2, 6, 10, 17, 27). Direct challenge of the middle ear bulla with bacteria is the method used for both animal models. Here, we wanted to test the possibility of protection following active immunization with LPD, PDm, and NTHi against an ear infection induced by direct challenge with 10^4 or 50 CFU of NTHi in the middle ear bullae of rats. Barenkamp showed that passive immunization of chinchillas with a hyperimmune serum raised against an NTHi strain elicited protection in all animals challenged with the homologous strain (6). In our study, on the fourth day of bacterial challenge 7 of 10 rats that had been systemically immunized with NTHi were protected from developing otitis media. By the eighth day, the remaining three rats had recovered. Immunization with LPD or PDm failed to protect rats from developing otitis media, but examination of ears on the eighth day showed the recovery rate to be higher among LPD-immunized rats than among PDm- or sham-immunized rats (5 of 10 versus 3 of 10 and 2 of 10, respectively). Also, middle ear specimens taken on the eighth day showed 3 of 10 LPD-immunized rats, 0 of 10 PDm-immunized rats, and 2 of 10 sham-immunized rats to be culture negative. With the chinchilla otitis model, others have shown systemic immunization with pili to be 50% protective against homologous challenge with 10 CFU of bacteria (22). In another study, 50% of chinchillas challenged with 10^3 CFU of NTHi after systemic immunization with high-molecular-weight adhesion proteins were found to be protected (5). Recently, in the chinchilla otitis model, systemic immunization with P6 was shown to induce a 48% recovery rate and a 51% reduction in the number of positive middle ear cultures 14 days after the bacterial challenge compared with sham immunization (10). There have been noteworthy differences between the chinchilla and rat otitis media models, mainly in the amount of NTHi inoculum necessary to induce purulent otitis (10^4 CFU in the rat model versus 10^1 to 10^3 CFU in the chinchilla model). To assess the importance of inoculum size in the protection studies, we challenged separate groups of LPD-immunized or PBS-injected rats with 200 times fewer (50 CFU) bacteria. Decreasing the bacterial inoculum size did not prevent the development of AOM in LPD-immunized rats, but the recovery rate was 45%, compared with 14% for the PBS-injected rats. Middle ear specimens from 36% of the LPD-immunized rats and 57% of the PBS-injected rats were culture positive for NTHi.

Owing to the fact that direct challenge of the middle ear with bacteria is not the natural route by which NTHi induces otitis media in humans, efforts to design new animal models that will better mimic the natural course of infection have been made. Nasopharyngeal challenge followed by the application of negative pressure in order to aspirate NTHi up into the middle ear through the eustachian tube is an approach that has been used with chinchillas (17). Another method where a natural route of infection is used includes the addition of adenovirus prior to NTHi challenge (34). Both of these methods are based on mucosal colonization in the pharynx prior to migration of bacteria into the middle ear. Mucosal antibodies (secretory IgA) have been shown to inhibit the adherence of bacteria to nasopharyngeal epithelial cells (24). Introduction of antigen by the mucosal route appears to be an effective way to induce a mucosal immune response. Kurono and coworkers have shown that mice given peroral liposomes containing inactivated NTHi develop high levels of salivary anti-NTHi IgA antibodies and that nasopharyngeal colonization of these mice by NTHi is significantly decreased (24). In a separate study, we showed intranasal or peroral immunization of rats to induce salivary IgA antibodies to protein D (2). Immunization with protein D via the mucosal or mucosal-systemic route to test the effect of a protein D vaccine in an animal model that mimics the natural course of infection (i.e., nasopharyngeal challenge with NTHi) might prove a better method for testing protein D as a vaccine against NTHi-induced otitis media.

The effects of protein carriers in conjugate Hib vaccines on the immune response to PRP may vary. PRP-OMPC vaccine has been shown to elicit early anti-PRP antibodies, while

PRP-T and PRP-CRM_{197} (mutant diphtheria toxin) yield higher IgG levels after two booster doses (9, 16). Furthermore, OMPC has been shown to act as an adjuvant for concurrently administered antigens, probably through its mitogenic effect on lymphocytes and macrophages (25, 26). These vaccines induce good immune responses to PRP only and not against other *H. influenzae* components. Therefore, their effect is limited to Hib strains, and they provide no protection against NTHi infections. Antibodies to membrane proteins of *H. influenzae* are involved in protection not only against NTHi infections but also against Hib infections, and they have been suggested as vaccine candidates (7, 13, 15, 21, 25). Our earlier studies with protein D have shown that protein D is present on the membranes of both encapsulated and unencapsulated strains (1), that it is a target for serum antibodies of rats recovering from experimentally induced AOM (27), that local or systemic immunization with bacteria bearing protein D induces local or systemic antibodies to protein D (2), and that serum antibodies to PDm are bactericidal to NTHi strains (3). In this study, the acylated form (LPD) of protein D was more immunogenic than was the nonacylated form (PDm) and induced serum antibodies with higher bactericidal activity than did PDm. Recently, Snapper and coworkers demonstrated that when LPD is incubated in a B-cell culture with anti-IgD-antibody-conjugated dextran molecules, a multivalent antigen receptor cross-linking signal, it enhances costimulation of immunoglobulin secretion and cellular proliferation (33). Multivalent antigen receptor cross-linking represents an in vitro polyclonal stimulation model of T-cell-independent type 2 antigens such as bacterial polysaccharides. In their report, Snapper and colleagues suggested that, together with bacterial polysaccharides, lipoproteins of encapsulated bacteria deliver costimulatory signals to B cells which upregulate antibody responses to polysaccharides.

In view of these data, we synthesized LPD- and PDm-conjugated PRP vaccines to assess the in vivo adjuvant and carrier effects of protein D. Screening of immune sera from rats immunized with these vaccines and from PRP-T-immunized rats showed LPD-containing vaccines to yield significantly higher levels of serum anti-PRP IgG and IgA than the other vaccines tested. The amount of LPD conjugated to PRP appears to determine the level of anti-PRP antibodies induced. PRP-T-immunized rats had the third-highest antibody levels, after the second group of LPD-immunized rats. The level of antibodies to PRP seems to be a determinant of bactericidal activity, as correlation was found to exist between the values for the two variables measured in serum. Immune sera from LPD- and PDm-immunized animals, containing high levels of anti-LPD or anti-PDm antibodies, did not have bactericidal activity against Hib strain Minn A, although antibodies in these sera detected protein D on a Hib strain Minn A OMP preparation in a Western blot assay. These results suggest that antibodies to protein D in the sera of animals immunized with LPD- or PDm-conjugated PRP vaccines do not play a role in the biological activities of these sera against Hib strain Minn A. The difference in biological activity observed between PRP-T vaccine and the protein D-conjugated PRP vaccines may be due to differences in the level of antibodies to PRP and to possible differences in the quality of antibodies induced by these vaccines.

Pooled sera from animals in group A (immunized with PRP-LPD) and group C (immunized with PRP-PDm) were also tested for bactericidal activity against NTHi strain 3655. The amount of protein D given with these vaccinations was approximately 17 times lower than in LPD or PDm immunizations with Freund's adjuvant, and the resulting level of antibody was 11 to 5 times lower. However, both groups manifested bactericidal activity against NTHi strain 3655. Interestingly, sera of animals immunized with LPD-conjugated vaccine had significantly higher bactericidal activities than sera of animals immunized with PDm-conjugated vaccine; 50%-killing dilutions were 1:8 and 1:2, respectively. This difference could be due to qualitative differences between the antibodies in the two groups, since sera from both detected a single 42-kDa band corresponding to protein D in a Western blot assay against NTHi strain 3655 OMP preparations (data not shown) and there was no significant difference in the amount of LPD antibodies.

Although the majority (90%) of cases of *H. influenzae*-induced AOM are due to NTHi strains, Hib strains are reported to cause AOM in 10% of cases (8). We have shown that AOM can be induced experimentally in rats both by NTHi and by Hib strains (27). In this study, we chose to use the Hib-induced-AOM model because this model would allow us to assess the protective effect of active immunization with the PRP conjugate vaccines. Studies with high-risk children have also shown that sera from individuals hyperimmunized with bacterial polysaccharide are preventive against AOM caused by encapsulated bacteria when those sera are administered systemically (32). Vaccination against another type of encapsulated bacterium, pneumococci, was found to be effective in protecting humans and chinchillas from AOM (14, 23). In this study, our attempt to evaluate the potential for protection with the protein D conjugate vaccines and PRP-T in an experimental rat model of Hib strain Minn A-induced otitis media showed that the PRP-LPD conjugate vaccine with higher LPD content yielded the best protection rate of all six alternatives.

To sum up, we showed that immunization with LPD yielded higher antibody levels and higher levels of bactericidal activity against NTHi than did immunization with PDm. Although systemic immunization with LPD and PDm did not protect against experimental otitis media induced by direct challenge in the middle ear with NTHi, LPD immunization might affect the course of otitis media by promoting faster recovery.

Immunization of rats with LPD- or PDm-conjugated PRP vaccines showed higher LPD content to be associated with higher serum PRP antibody concentrations. Furthermore, serum anti-PRP antibody levels correlated with both the level of bactericidal activity and the protection rates in rats with AOM experimentally induced with Hib strain Minn A. Lastly, LPD-conjugated PRP vaccine induced serum antibodies with significantly higher bactericidal activity against NTHi strain 3655 than that of antibodies induced by PDm-conjugated PRP vaccine.

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REFERENCES

1. **Akkoyunlu, M., M. Ruan, and A. Forsgren.** 1991. Distribution of protein D, an immunoglobulin D-binding protein, in *Haemophilus* strains. Infect. Immun. **59:**1231–1238.
2. **Akkoyunlu, M., and A. Forsgren.** 1996. Local and systemic antibody levels against protein D of *Haemophilus influenzae* following infection and immunization in rats. APMIS **104:**709–717.
3. **Akkoyunlu, M., H. Janson, M. Ruan, and A. Forsgren.** 1996. Biological activity of serum antibodies to a nonacylated form of lipoprotein D of

Haemophilus influenzae. Infect. Immun. **64:**4586–4592.
4. **Ashwell, G.** 1957. Colorimetric analysis of sugars. Methods Enzymol. **3:**73–105.
5. **Barenkamp, S. J.** 1996. Immunization with high-molecular-weight adhesion proteins of nontypeable *Haemophilus influenzae* modifies experimental otitis media in chinchillas. Infect. Immun. **64:**1246–1251.
6. **Barenkamp, S. J.** 1986. Protection by serum antibodies in experimental nontypable *Haemophilus influenzae* otitis media. Infect. Immun. **52:**572–578.
7. **Barenkamp, S. J., and F. F. Fodor.** 1990. Development of serum bactericidal activity following nontypable *Haemophilus influenzae* acute otitis media. Pediatr. Infect. Dis. J. **9:**333–339.
8. **Dabernat, H., C. Delmas, C. Rich, V. Livrelli, and B. Joly.** 1988. Characterization of *Haemophilus influenzae* isolated from patients with otitis media. Eur. J. Clin. Microbiol. Infect. Dis. **7:**219–225.
9. **Decker, M. D., K. M. Edwards, R. Bradley, and P. Palmer.** 1992. Comparative trial in infants of four conjugate *Haemophilus influenzae* type b vaccines. J. Pediatr. **120:**184–189.
10. **DeMaria, T. F., D. M. Murwin, and E. R. Leake.** 1996. Immunization with outer membrane protein P6 from nontypeable *Haemophilus influenzae* induces bactericidal antibody and affords protection in the chinchilla model of otitis media. Infect. Immun. **64:**5187–5192.
11. **Erdile, L. F., M.-A. Brandt, D. J. Warakomski, G. J. Westrack, A. Sadziene, A. G. Barbour, and J. P. Mays.** 1993. Role of attached lipid in immunogenicity of *Borrelia burgdorferi* OspA. Infect. Immun. **61:**81–90.
12. **Evans, F. O. J., J. B. Sydnor, W. E. C. Moore, J. L. Manwaring, A. H. Brill, R. T. Jackson, S. Hanna, J. S. Skaar, L. V. Holdeman, G. S. Fitz-Hugh, M. A. Sande, and J. M. Gwaltney, Jr.** 1975. Sinusitis of the maxillary antrum. N. Engl. J. Med. **293:**735–739.
13. **Faden, H., L. Brodsky, J. Bernstein, J. Stanievich, D. Krystofik, C. Shuff, J. J. Hong, and P. L. Ogra.** 1989. Otitis media in children. I. The systemic immune response to nontypeable *Haemophilus influenzae*. J. Infect. Dis. **160:**999–1004.
14. **Giebnik, G.** 1981. The pathogenesis of pneumococcal otitis media in chinchillas and the efficacy of vaccination in prophylaxis. Rev. Infect. Dis. **3:**342–353.
15. **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.
16. **Granoff, D. M., E. L. Anderson, M. T. Osterholm, S. J. Holmes, J. E. McHugh, R. B. Belshe, F. Medley, and T. V. Murphy.** 1992. Differences in the immunogenicity of three *Haemophilus influenzae* type b conjugate vaccines in infants. J. Pediatr. **121:**187–194.
17. **Green, A. B., W. J. Doyle, and J. L. Cowell.** 1994. Chinchilla model of experimental otitis media for study of nontypable *Haemophilus influenzae* vaccine efficacy. Methods Enzymol. **235:**59–68.
18. **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.
19. **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* [sic]. Infect. Immun. **58:**3272–3278.
20. **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.
21. **Gulig, P. A., G. H. McCracken, Jr., C. F. Frisch, K. H. Johnston, and E. J. Hansen.** 1982. Antibody response of infants to cell surface-exposed outer membrane proteins of *Haemophilus influenzae* type b after systemic *Haemophilus* disease. Infect. Immun. **37:**82–88.
22. **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:**62–65.
23. **Kleine, J. O.** 1989. Pneumococcal otitis media vaccine trials. Pediatr. Infect. Dis. J. **8:**82–83.
24. **Kurono, Y., K. Shimamura, and G. Mogi.** 1992. Inhibition of nasopharyngeal colonization of *Haemophilus influenzae* by oral immunization. Ann. Otol. Rhinol. Laryngol. **101:**11–15.
25. **Loeb, M. R., and D. H. Smith.** 1982. Human antibody response to individual outer membrane proteins of *Haemophilus influenzae* type b. Infect. Immun. **37:**1032–1036.
26. **Mäkelä, P. H., H. Pelton, H. Käythy, H. Jouismies, E. Rusolahti, A. Sivonen, and O. V. Renkonen.** 1977. Polysaccharide vaccines of group A *Neisseria meningitidis* and *Haemophilus influenzae* type b: a field trial in Finland. J. Infect. Dis. **136:**43–50.
27. **Melhus, Å., A. Hermansson, M. Akkoyunlu, K. Prellner, and A. Forsgren.** 1995. Experimental recurrent otitis media induced by *Haemophilus influenzae*. Protection and serum antibodies. Am. J. Otolaryngol. **16:**383–390.
28. **Moxon, E. R., A. L. Smith, D. R. Averill, and D. H. Smith.** 1974. *Haemophilus influenzae* meningitis in infant rats after intranasal inoculation. J. Infect. Dis. **129:**154–162.
29. **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.
30. **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.
31. **Musher, D. M., K. R. Kubitshek, J. Crennan, and R. E. Baughn.** 1983. Pneumonia and acute tracheobronchitis due to *Haemophilus influenzae*. Ann. Intern. Med. **99:**444–450.
32. **Shurin, P. A., J. M. Rehmus, E. C. Johnson, C. D. Marchant, S. A. Carlin, D. M. Super, G. F. van Hare, P. K. Jones, D. M. Ambrosino, and G. R. Siber.** 1993. Bactericidal polysaccharide immune globulin for prophylaxis of acute otitis media in high-risk children. J. Pediatr. **123:**801–809.
33. **Snapper, C. M., F. R. Rosas, L. Jin, C. Wortham, M. T. Kehry, and J. J. Mond.** 1995. Bacterial lipoproteins may substitute for cytokines in the humoral immune response to T cell-independent type II antigens. J. Immunol. **155:**5582–5589.
34. **Suzuki, K., and L. O. Bakaletz.** 1994. Synergistic effect of adenovirus type 1 and nontypeable *Haemophilus influenzae* in a chinchilla model of experimental otitis media. Infect. Immun. **62:**1710–1718.
35. **Teele, T. W., J. O. Klein, B. Rosner, and Greater Boston Otitis Media Study Group.** 1989. Epidemiology of otitis media during the first seven years of life in children in greater Boston: a prospective, cohort study. J. Infect. Dis. **160:**83–94.
36. **Yamanaka, N., and H. Faden.** 1993. Antibody response to outer membrane protein of nontypable *Haemophilus influenzae* in otitis-prone children. J. Pediatr. **122:**212–218.

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