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Detoxified Lipooligosaccharide from Nontypeable *Haemophilus influenzae* Conjugated to Proteins Confers Protection against Otitis Media in Chinchillas

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Detoxified-lipooligosaccharide (dLOS)–protein conjugates from nontypeable *Haemophilus influenzae* (NTHi) elicited a significant rise of anti-LOS antibodies with bactericidal activity in rabbits (X.-X. Gu, C.-M. Tsai, T. Ueyama, S. J. Barenkamp, J. B. Robbins, and D. J. Lim, Infect. Immun. 64:4047–4053, 1996). In this study, we evaluated whether vaccination with the conjugates would protect against NTHi otitis media in chinchillas. Fifty-eight chinchillas received three subcutaneous or intramuscular injections of dLOS-conjugated tetanus toxoid, dLOS-conjugated high-molecular-weight proteins from NTHi, or saline (control) in Freund's adjuvant and then were challenged by intrabullar inoculation with 140 CFU of NTHi. All vaccinated animals responded with elevated serum titers of anti-LOS antibody, and 49% (19 of 39) demonstrated bactericidal activity against the homologous strain. Otitis media with culture-positive NTHi effusions developed in all 19 controls and 56% (22 of 39) of the vaccinated animals during a period of 21 days ($P < 0.001$). Bacterial counts of the middle ear effusions were lower in the vaccine groups than in the controls ($P < 0.01$). The incidences of infection in the unchallenged ear or inner ear were 26 or 28% in the vaccine groups and 53 or 58% in the controls ($P < 0.05$). The signs of infection observed by otoscopy were less severe in the vaccine groups than in the controls. There was no significant difference between the two vaccine groups. These data indicate that active immunization with LOS-based conjugates reduces the incidence of NTHi-induced otitis media.

Nontypeable *Haemophilus influenzae* (NTHi) is an important cause of otitis media (OM) in children and respiratory tract diseases in adults (28, 32, 34). Prevention of NTHi OM is sought since it is a common childhood disease and about 10% of OM patients suffer persistent middle ear (ME) infection and mild-to-moderate hearing loss (17). The annual cost of the medical and surgical treatment of OM in the United States is between $3 billion and $4 billion (4). Furthermore, inappropriate antibiotic treatment of OM encourages the emergence of multidrug-resistant strains of bacteria, underscoring the importance of preventing OM (4).

Development of a vaccine for NTHi has been difficult because of a lack of understanding of which antigens confer immunity, as well as a lack of clinical studies identifying an in vitro correlate of immunity to NTHi infection. Host immunity is believed to play a role in OM (15, 37, 40), since serum and ME effusion antibodies directed against NTHi develop during the course of infection (5, 8, 14, 15, 39). Bactericidal antibodies are associated with reduced numbers of NTHi in ME effusion (14) and more rapid resolution of infection (8, 36, 37).

Surface antigens of NTHi, such as outer membrane proteins, pili or fimbriae, and lipooligosaccharide (LOS), have been evaluated as vaccine candidates (1, 9, 11, 22, 26, 33, 38). LOS, a major surface-exposed antigen of NTHi, is a virulence factor. Human antibodies and mouse monoclonal antibodies against LOS have shown bactericidal activity in vitro (3, 27). A mouse LOS monoclonal antibody was opsonic and enhanced bacterial clearance in a murine pulmonary challenge model (30).

We prepared two detoxified-LOS (dLOS)–protein conjugates from NTHi strain 9274, dLOS-TT (tetanus toxoid) and dLOS-HMP (high-molecular-weight proteins from NTHi), and demonstrated that the conjugates were immunogenic in animal models and induced bactericidal antibodies against the homologous strain and a heterologous strain in rabbits (26). In this study, we determined whether the dLOS-protein conjugates elicit immunity against NTHi otitis media in a chinchilla model.

MATERIALS AND METHODS

Conjugate vaccines. NTHi strain 9274, a clinical isolate from ME fluids of a patient with OM, was provided by M. A. Apicella, University of Iowa. Bacterial growth, purification of LOS from strain 9274 (24, 25), detoxification of the LOS, conjugation of dLOS to TT or HMP from NTHi strain 12 (1), and characterization of dLOS-TT and dLOS-HMP have been described previously (26). Both conjugates were analyzed for carbohydrate and protein by using dLOS and bovine serum albumin as standards (26). The composition of dLOS-TT was 150 μg of dLOS per ml and 231 μg of TT per ml with a molar ratio of dLOS to TT of 32:1, while the composition of dLOS-HMP was 152 μg of dLOS per ml and 223 μg of HMP per ml with a molar ratio of 27:1, respectively.

Experimental scheme. This was a randomized, blind, controlled study of active prevention of acute OM caused by NTHi in chinchillas approved by the National Institute of Neurological Disorders and Stroke-National Institute on Deafness and Other Communication Disorders Animal Care and Use Committee National Institutes of Health. A total of 58 outbred, healthy, adult chinchillas weighing between 400 and 600 g were purchased from Moulton Chinchilla Ranch, Rochester, Minn., and housed in separate cages. All animals were kept in quarantine for 1 week to acclimate them to the laboratory.

The 58 animals were randomly assigned to three groups (saline [$n = 19$], dLOS-TT [$n = 20$], and dLOS-HMP [$n = 19$]), and a blood sample was collected from the transverse venous sinus of each chinchilla (7) to assess antibody levels. Three days later, the animals were immunized with three doses of the two

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conjugates or saline (as a control) at 4-week intervals and challenged by injection of 140 CFU of strain 9274 into the right ME 14 days after the last immunization. Both ears were examined daily by otoscopy for evidence of acute OM for 21 days postchallenge. On days 3, 7, 14, and 21 postchallenge, four or five animals from each group were sacrificed by ketamine injection followed by cervical dislocation and the ME fluids from both ears were cultured for bacterial counting. Blood samples were also collected from all of the chinchillas 14 days after the first and second immunizations, 10 days after the third immunization, and before sacrifice. The animals were anesthetized with ketamine-HCl (30 mg/kg of body weight given intramuscularly) prior to all operative procedures.

Immunization. Three groups of chinchillas were injected with 25 μg of dLOS-TT, dLOS-HMP (dLOS content), or saline in 0.3 ml (0.15 ml injected intramuscularly into the right rear leg and 0.15 ml injected subcutaneously into the back) at each of the three immunizations. The immunogens including saline had been emulsified 1:1 in complete Freund's adjuvant for the first injection and in incomplete Freund's adjuvant (Difco, Detroit, Mich.) for the second and third injections.

Bacterial growth and ME challenge. The bacteria were recovered from Greave's solution stocks by transferring a loopful of thawed organisms to a chocolate agar plate and incubated at 37°C under a 5% CO_2 atmosphere for 16 h. Five to ten colonies were transferred to 50 ml of 3% brain heart infusion broth supplemented with NAD (5 μg/ml) and hemin (2 μg/ml) (Sigma Chemical Co., St. Louis, Mo.) in a 250-ml Erlenmeyer flask. Growth proceeded for 4 to 6 h at 37°C with shaking at 150 rpm (G25 shaker; New Brunswick Scientific Co., Edison, N.J.). Bacteria in the mid-log growth phase with an A_{600} of 0.5 to 0.6 were harvested by centrifugation (3,360 × *g*) at 4°C for 10 min and then washed twice with phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin and $CaCl_2$ and $MgCl_2$ at final concentrations of 0.15 and 0.5 mM, respectively (1). The washed bacteria were maintained at 4°C before being used for challenge. Fifty-eight chinchillas were anesthetized with ketamine, the area over the superior bulla was shaved and cleaned, and then 0.2 ml of approximately 140 CFU of bacteria was injected aseptically via puncture of the superior aspect of the right cephalic bulla (13, 18) with a 25-gauge needle attached to a 1-ml syringe. The inoculum (>5 100% effective doses) was predetermined to induce symptomatic OM in all of the challenged chinchillas within 48 h.

Otoscopic examination. Otoscopic examination was performed by two investigators daily for 21 days postchallenge. A diagnosis of acute OM was made by rating ME pathology on a scale of 0 to 4+ as described by Giebink et al. (20) and Green et al. (21) with modifications. A rating of 0 indicates a normal ear; 1+ indicates evidence of congestion along the edges of the tympanic membrane (TM) but the TM was normal; 2+ indicates congestive TM, opaque TM, or any minimal pathological changes on the TM such as slight retraction, slight erythema, and slight bulging; 3+ indicates evidence of moderate pathological changes in the TM such as severe bulging and/or erythema but the TM could be moved partially by the aspiration; and 4+ indicates substantial ME effusions with signs of severe TM pathologies such as an observable air-fluid level, fluids with a fixed TM, or evidence of perforation such as a discharge. A mean index of otoscopic examinations was derived daily by adding the scores from all of the surviving chinchillas of each group and dividing the result by the number of surviving chinchillas in each group.

A diagnosis of labyrinthitis (or inner ear infection) was made when the animal exhibited head tilting or circling behavior (20, 37).

ME effusions. On days 3, 7, 14, and 21 postchallenge, four or five chinchillas in each group were sacrificed. Their superior bullae were opened, and ME effusions were aspirated from the inferior bullae with a 23-gauge needle with a suitable angle. Following aspiration, the MEs were washed with 0.5 ml of sterile PBS and the volume, color, and consistency of the ME effusions were recorded. An aliquot of ME effusions and their serial dilutions were plated on chocolate agar for a quantitative count of NTHi. All procedures were performed under sterile conditions (21).

ELISA. Serum titers of antibody to NTHi 9274 LOS were determined by enzyme-linked immunosorbent assay (ELISA) (1, 26). Briefly, 96-well plates (Immuno I) were coated with 9274 LOS (10 μg/ml) in PBS (pH 7.4) with 0.1 mM $MgCl_2$ and incubated overnight at 4°C. On the following day, the plates were blocked with 5% fetal calf serum in PBS for 1 h. The diluted sera were added and the mixture was incubated for 2 h. To detect immunoglobulin G (IgG) and IgM, rabbit anti-chinchilla IgG and IgM sera (1:500) (1), followed by alkaline phosphatase (AP)-conjugated goat anti-rabbit IgG and IgM (1:2,000) (Sigma), were added with 1 h of incubation for each. To detect IgG, protein A-AP conjugate (Sigma) was added with 1 h of incubation. All steps were performed at room temperature, and PBS–0.05% Tween 20 was used in five washings between steps. Diluents for sera and conjugates were 5% fetal calf serum–PBS–0.05% Tween 20. After the AP substrate was added and the mixture was incubated for 30 min, the reactions were read with a microplate autoreader to determine the A_{405}. A chinchilla antiserum against dLOS-TT was used as a positive control for each plate. Negative controls included buffer and AP conjugate. All negative controls and preimmunization sera gave optical density readings of less than 0.3 for IgG and IgM or 0.1 for IgG. Chinchilla serum antibodies to TT or HMP were measured by ELISA as described above with TT or HMP as the coating antigen (5 μg/ml in 0.1 M Tris buffer, pH 9.8).

Bactericidal assay. Chinchilla pre- and postimmune sera (after the third injection) were inactivated at 56°C for 30 min and tested for bactericidal activity against homologous strain 9274 (26). Briefly, a fivefold dilution of the initial sera and then twofold serial dilutions were made, so that 50 μl of diluted sera was present in each well of a sterile 96-well plate. A 30-μl volume of a log-phase bacterial suspension (about 3×10^3 CFU/ml) was added, followed by 20 μl of infant rabbit serum as a source of complement. The plates were incubated at 37°C for 45 min, and 50 μl of the mixture was transferred from each well onto chocolate agar plates. The plates were incubated at 37°C in 5% CO_2 overnight, and the colonies were counted. The highest serum dilution resulting in >50% killing was expressed as the reciprocal bactericidal titer.

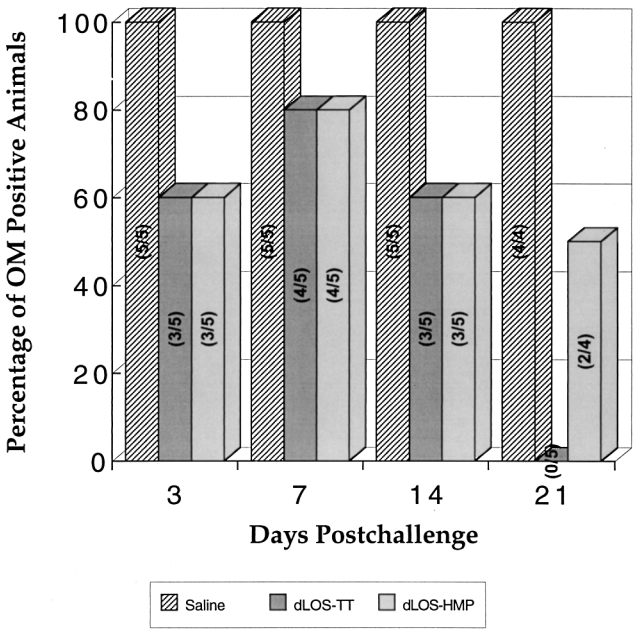

FIG. 1. Percentage of chinchillas in each group with OM with culture-positive effusions up to 21 days postchallenge. The values in parentheses represent the number of infected animals/total number of animals. Comparisons: on day 21, saline versus dLOS-TT ($P < 0.01$) and saline versus the two conjugates ($P < 0.05$); during the whole course, saline versus dLOS-TT or the two conjugates ($P < 0.001$) and saline versus dLOS-HMP ($P < 0.01$). There was no statistically significant difference between dLOS-TT and dLOS-HMP.

Statistical analysis. Antibody levels were expressed as the geometric mean (GM) ELISA titers (reciprocal) of *n* independent observations ± the standard deviation (SD). The ME bacterial densities were expressed as the GM CFU of *n* independent observations ± the SD. The otoscopic examination outcome was expressed as the mean of *n* independent observations ± the SD at each time point. Differences between the control and conjugate groups were determined by using the Student *t* test. Fisher's exact test (one tailed) was employed to compare the proportions of infected animals found in the control and conjugate groups.

RESULTS

Active protection in the chinchilla OM model. All controls developed OM with culture-positive NTHi effusions up to 21 days postchallenge (Fig. 1). In contrast, 60% of chinchillas from both conjugate groups developed OM on day 3, 80% did so on day 7, and 60% did so on day 14. On day 21, no animals in the dLOS-TT group and only 50% of the animals in the dLOS-HMP group showed OM with effusions. The incidence of OM was significantly lower in the dLOS-TT group than in the controls on day 21 (0 of 5 versus 4 of 4; $P < 0.01$). The incidence of OM was also lower in the conjugate groups than in the controls on day 21 (2 of 9 versus 4 of 4; $P < 0.05$) and over the whole course (22 of 39 versus 19 of 19; $P < 0.001$). There was no significant difference between the dLOS-TT and dLOS-HMP groups.

Table 1 shows the culture positivity rates of ME fluids from both the challenged and the unchallenged ears of each immunized group on days 3, 7, 14, and 21 postchallenge. The inci-

TABLE 1. Culture-positive ME effusions from immunized chinchillas challenged with NTHi strain 9274[a]

Postchallenge day	No. of positive ME effusions/no. of animals			
	Saline		Conjugates (dLOS-TT, dLOS-HMP)	
	Right	Left	Right	Left
3	5/5	3/5	6/10 (3/5, 3/5)	3/10 (1/5, 2/5)
7	5/5	4/5	8/10 (4/5, 4/5)	5/10 (3/5, 2/5)
14	5/5	1/5	6/10 (3/5, 3/5)	2/10 (1/5, 1/5)
21	4/4*	2/4†	2/9* (0/5, 2/4)	0/9† (0/5, 0/4)
Total	19/19**	10/19*	22/39**	10/39*

[a] Right represents the challenged ear, and left represents the unchallenged ear. Saline group versus conjugate-immunized group: †, $P = 0.07$ to 0.08; *, $P < 0.05$; **, $P < 0.001$. There was no statistically significant difference between the dLOS-TT and dLOS-HMP groups.

dence of culture-positive effusions from challenged (right) ears was identical to that of individual animals. The incidences of culture-positive effusions from unchallenged (left) ears were 0% in the conjugate groups and 50% in the controls on day 21 (0 of 9 versus 2 of 4; $P = 0.077$), 26% in the conjugate groups, and 53% in the controls during the whole course (10 of 39 versus 10 of 19; $P < 0.05$). The incidences of inner ear infection as manifested by head tilting or dysequilibrium were 28% in the conjugate groups and 58% in the controls (11 of 39 versus 11 of 19; $P < 0.05$).

Bacterial counts of the ME effusions from the right (challenged) ears of the animals showed significant differences between the conjugate and control groups (Fig. 2). All of the controls contained bacteria (mean count, 2.63×10^7 CFU/ml) throughout the 21-day course. In contrast, all of the dLOS-TT- and dLOS-HMP-immunized animals had lower mean counts (1.2×10^3 and 1.0×10^4 CFU/ml, respectively). Conjugate-immunized animals had significantly lower bacterial counts than did controls on days 3, 7, 14, and 21 ($P < 0.01$). Bacterial counts of ME effusions from left ears showed a pattern similar to that of those from right ears (data not shown).

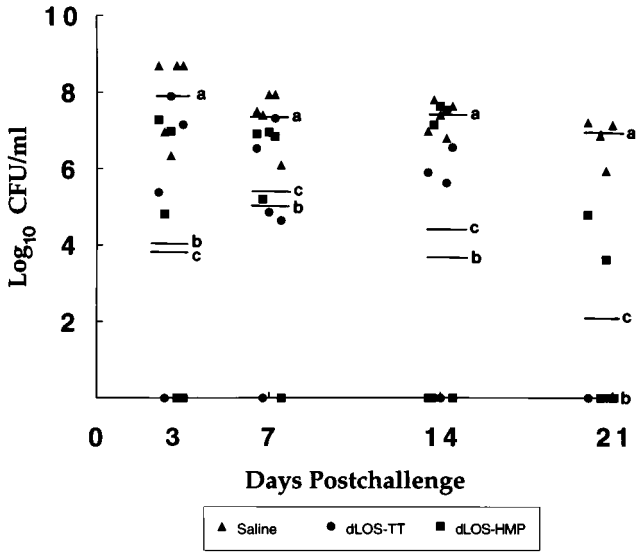

FIG. 2. NTHi culture counts recovered from ME effusions or ME washing fluids from the right (challenged) ears of 58 chinchillas at 3, 7, 14, and 21 days postchallenge. a, b, and c at each time point represent the GM CFU of the saline, dLOS-TT, and dLOS-HMP groups, respectively. Saline versus dLOS-TT or dLOS-HMP at each time point, $P < 0.01$.

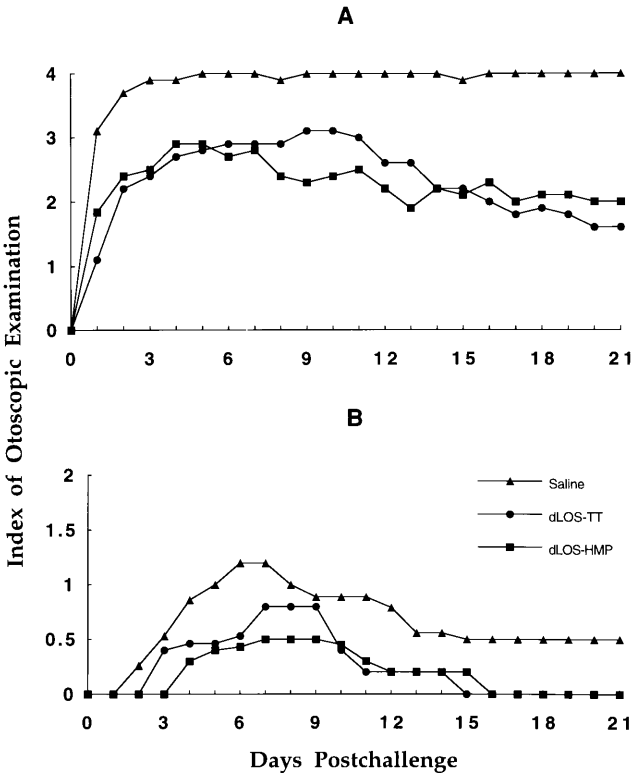

FIG. 3. Indexes of otoscopic examination of chinchillas from different groups up to 21 days postchallenge. The curve on each day is the mean index of each group, rated on a scale of 0 (normal ear) to 4+ (severe otitis media with effusions), as determined by otoscopy (A, right [challenged]; B, left [control]). The mean index from the three immunization groups, saline, dLOS-TT, and dLOS-HMP, was obtained as follows: 19, 20, and 19 chinchillas, respectively, were scored up to postchallenge day 3; 14, 15, and 14 chinchillas were scored from day 4 to day 7; 9, 10, and 9 chinchillas were scored from day 8 to day 14; and 4, 5, and 4 chinchillas were scored from day 15 to day 21 postchallenge.

Otoscopic examination of the right (challenged) ME showed signs of acute OM in the majority of animals on day 1 postchallenge; however, more severe signs (higher indexes) of infection were observed in the controls (3.1 ± 0.8) than in the dLOS-TT (1.1 ± 1.2) or dLOS-HMP (1.8 ± 1.4) group (Fig. 3A). By day 2, approximately 90% of the controls showed severe signs of OM with a rating of 4+ while only about 40% of conjugate-immunized animals had such severe OM. On day 4, all controls showed severe signs of infection (4+) persisting until day 21. In contrast, only about 55% of conjugate-immunized animals showed severe signs (4+) by day 4 and the maximum percentage of animals rated at 4+ was 65% during days 5 to 11. By day 13, the conjugate-immunized animals showed clearing of infection and about 60% (dLOS-TT) and 50% (dLOS-HMP) of the animals recovered from infection by day 21. The mean indexes for the control, dLOS-TT, and dLOS-HMP groups were 4 ± 0, 1.6 ± 1.1, and 2 ± 1.6 on day 21 ($P < 0.001$). Similar patterns were also observed from the left (unchallenged) ears, although the signs were less severe and shorter in duration (Fig. 3B).

Serum antibodies. Three injections of saline did not elicit a rise of LOS antibodies in controls (Table 2). In contrast, both conjugates elicited significant levels of LOS antibodies with a 60- to 70-fold increase in IgG and IgM and a 20- to 40-fold

TABLE 2. Chinchilla antibody responses to NTHi strain 9274 LOS elicited by conjugates

Immunogen[a] and bleeding no.[b]	GM (± SD range) of ELISA titers	
	IgG + IgM	IgG
Saline		
1	33 (18–60)[c]	11 (8–16)
2	35 (20–61)	12 (8–18)
3	36 (22–61)	13 (8–20)
4	38 (24–60)	12 (8–18)
5	38 (24–60)	12 (8–18)
dLOS-TT		
1	30 (17–54)	16 (9–27)
2	1,950 (579–6,566)	726 (88–5,959)
3	3,771 (1,430–9,943)	1,951 (403–9,451)
4	3,984 (1,730–9,173)	3,378 (1,088–10,932)*
5	3,157 (1,248–8,902)	2,865 (822–9,989)
dLOS-HMP		
1	34 (17–67)	13 (8–20)
2	2,431 (588–10,044)	321 (40–2,571)
3	3,643 (1,069–12,431)	764 (120–4,853)
4	2,430 (567–10,418)	1,214 (174–8,490)**
5	2,292 (597–8,972)	1,286 (237–6,978)

[a] Fifty-eight chinchillas were immunized subcutaneously and intramuscularly with 3 doses of saline ($n = 19$), dLOS-TT ($n = 20$), or dLOS-HMP ($n = 19$) at 4-week intervals.

[b] Blood samples were collected from all chinchillas before immunization (no. 1), 2 weeks after the first and second immunizations (no. 2 and 3), 10 days after the third immunization (no. 4), and before sacrifice (no. 5).

[c] Saline-immunized sera or preimmunization sera versus conjugate-immunized sera: $P < 0.001$. There is no significant difference between dLOS-TT and dLOS-HMP, except in the IgG titers 10 days after the third immunization (* versus**, $P = 0.0506$).

TABLE 3. Chinchilla IgG antibody responses to TT and HMP elicited by conjugates

Immunogen[a] and bleeding no.[b]	GM (± SD range) of ELISA titers	
	TT	HMP
Saline		
1	21 (14–35)[c]	17 (14–44)
2	22 (17–29)	26 (19–57)
3	25 (16–37)	22 (14–51)
4	24 (19–41)	31 (17–56)
5	26 (16–34)	24 (19–50)
dLOS-TT		
1	26 (18–37)	ND[d]
2	7,665 (2,177–26,990)	ND
3	65,313 (25,609–166,571)	ND
4	81,358 (40,243–164,475)	ND
5	55,387 (20,375–150,557)	ND
dLOS-HMP		
1	ND	34 (22–54)
2	ND	48,638 (16,007–147,741)
3	ND	97,342 (47,490–199,526)
4	ND	163,757 (99,609–269,215)
5	ND	183,865 (121,815–277,524)

[a] See Table 2, footnote *a*.

[b] See Table 2, footnote *b*.

[c] Saline-immunized sera or preimmunization sera versus conjugate-immunized sera: $P < 0.001$.

[d] ND, not done.

increase in IgG after one injection. There was an about 100-fold increase in IgG and IgM and a 100- to 200-fold increase in IgG after three injections ($P < 0.001$). IgG levels elicited by dLOS-TT were higher than those elicited by dLOS-HMP after three injections (Table 2, bleeding 4; $P = 0.0506$). There was an inverse correlation between LOS antibody levels and bacterial counts from ME fluids among the 58 animals ($r = -0.298$; $P = 0.023$).

Table 3 shows the antibody responses to TT or HMP. Saline did not elicit TT or HMP antibodies, while both conjugates elicited significant levels of protein antibodies (IgG) with an approximately 300- to 1,400-fold increase after one injection and a 3,000- to 4,000-fold increase after three injections ($P < 0.001$). Most of the protein antibodies detected were IgG because the levels of IgG and IgM were similar to that of IgG alone by ELISA (data not shown).

Serum bactericidal activity. The sera of all of the chinchillas before immunization and those of the controls had no bactericidal activity. In contrast, 45 or 53% of dLOS-TT- or dLOS-HMP-immunized sera showed bactericidal activity against strain 9274 with mean titers of 1:43 and 1:46, respectively (Table 4). The titers of all bactericidal-antibody-positive animals ranged from 1:10 to 1:160. There was a correlation between the bactericidal-antibody titers and LOS antibody levels (ELISA) among the 58 animals ($r = 0.32$; $P = 0.016$).

DISCUSSION

Chinchilla, gerbil, and rat models have been developed to study OM (16, 21, 31), and among them, chinchillas provide the best available model for NTHi OM (19). Direct challenge of the ME with NTHi is more stringent and reliable than a nasopharyngeal challenge in terms of controlling the amount of bacteria introduced (1, 2, 21). Only the right ME was challenged, and the left ear served as a control. Infection of the left ear should mimic the route of infection whereby the organisms first colonize the nasopharynx and then enter through the eustachian tube (6). All chinchillas that received as little as 27 CFU of strain 9274 developed OM, and a positive bacterial culture in the ME effusions persisted for at least 21 days post-challenge (data not shown). Therefore, the challenge dose in this study was at least 5 100% effective doses.

We showed that immunization with either dLOS-TT or dLOS-HMP induced high titers of anti-LOS antibodies with bactericidal activity in serum, consistent with our previous study with rabbits (26). This vaccination protected against NTHi OM in chinchillas, as demonstrated by a reduced incidence and duration of NTHi culture-positive ME effusions, bacterial counts, signs of ME inflammation, and incidence of unchallenged (left)-ear OM and labyrinthitis.

TABLE 4. Bactericidal activities of chinchilla antisera elicited by conjugates

Immunogen(s)[a]	GM (± SD range) of bactericidal titers[b]		Positivity rate (%)
	Preimmunization sera	3rd-immunization sera	
Saline	<5[c]	5	0/19 (0)
dLOS-TT	<5	43 (18–104)	9/20 (45)
dLOS-HMP	<5	46 (17–128)	10/19 (53)
Conjugates[d]	<5	45 (18–114)	19/39 (49)

[a] See Table 2, footnote *a*.

[b] Reciprocal of the highest dilution of sera that showed >50% killing of NTHi strain 9274.

[c] Saline-immunized sera or preimmunization sera versus conjugate-immunized sera: $P < 0.001$.

[d] dLOS-TT group plus dLOS-HMP group.

The relationship between serum antibodies and immunity to OM caused by NTHi is not clear. Faden et al. (14) reported that serum antibodies are associated with strain-specific protection from NTHi OM in children. Children with NTHi OM lack bactericidal antibodies before their infection and develop strain-specific bactericidal antibodies following infection (14, 17, 37). In our study, all conjugate-immunized chinchillas developed anti-LOS antibodies and 49% of the sera showed bactericidal activity against the homologous strain. The serum LOS antibody titers measured by ELISA and the chinchilla ME bacterial counts were inversely correlated, and the serum LOS antibody titers were also significantly correlated with serum bactericidal activity titers. These data suggest that protection is induced by the LOS antibodies elicited by the dLOS-protein conjugates and the bactericidal antibodies play an important role in protection.

This study showed significant differences in the clinical signs of OM between the control and conjugate-immunized groups. Other studies with outer membrane proteins (23) or P6 (13) as immunogens showed no differences between vaccinated animals and controls. Reduction of severity may be due to the neutralization effect of anti-LOS antibodies (IgG) elicited by the dLOS-protein conjugates in the ME cavity since ME inflammation may be induced by endotoxin (LOS) and/or peptidoglycan remaining in the ME after viable bacteria have been eliminated (12, 29, 42).

HMPs are adhesins from NTHi (41), and immunization with HMP alone showed protection in the chinchilla OM model (1). In our study, no additional protection was generated by dLOS-HMP although dLOS-HMP elicited high titers of serum HMP antibodies and the challenge strain 9274 expressed the HMP by Western blotting with a HMP monoclonal antibody (1) (unpublished data). The lack of additional protection may be due to the low degree of expression of HMP in the challenge strain, modification of the protective epitopes of HMP during chemical conjugation, the conformation change which occurs when HMP is used as a carrier protein to elicit antibodies, etc.

In summary, our data demonstrated that dLOS-TT and dLOS-HMP were immunogenic in chinchillas and induced high titers of anti-LOS antibodies with bactericidal activity in serum. Vaccination with the two conjugates resulted in protection against the experimental OM in chinchillas. Although NTHi LOSs are antigenically heterogeneous (10, 35), the LOS used for the conjugates was highly cross-reactive to NTHi clinical isolates because the rabbit bactericidal antisera elicited by the conjugates (26) can bind to the majority of NTHi clinical isolates tested by ELISA and also showed bactericidal activity against most of them (unpublished data). In addition, bactericidal and opsonophagocytic mouse monoclonal antibodies generated by this LOS can also bind to the majority of the NTHi clinical isolates (27). On the basis of these, the detoxified LOS-protein conjugates are promising vaccine candidates for prevention of OM caused by NTHi.

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