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Synthesis, Characterization, and Immunologic Properties of Detoxified Lipooligosaccharide from Nontypeable *Haemophilus influenzae* Conjugated to Proteins

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Nontypeable *Haemophilus influenzae* (NTHi) is an important cause of otitis media in children and of pneumonitis in adults with depressed resistance. Lipooligosaccharide (LOS) is a major surface antigen of NTHi and elicits bactericidal and opsonic antibodies. We prepared detoxified LOS (dLOS) protein conjugates from NTHi for use as experimental vaccines. LOS from NTHi 9274 was treated with anhydrous hydrazine and had its toxicity reduced to clinically acceptable levels. dLOS was bound to tetanus toxoid (TT) or high-molecular-weight proteins (HMPs) from NTHi through a linker of adipic acid dihydrazide to form dLOS-TT or dLOS-HMP. The molar ratio of the dLOS to protein carriers ranged from 26:1 to 50:1. The antigenicity of the conjugates was similar to that of the LOS alone as determined by double immunodiffusion. Subcutaneous or intramuscular injection of the conjugates elicited a 28- to 486-fold rise in the level of immunoglobulin G antibodies in mice to the homologous LOS after two or three injections and a 169- to 243-fold rise in the level of immunoglobulin G antibodies in rabbits after two injections. The immunogenicity of the conjugates in mice and rabbits was enhanced by formulation with monophosphoryl lipid A plus trehalose dimycolate. In rabbits, conjugate-induced LOS antibodies induced complement-mediated bactericidal activity against the homologous strain 9274 and prototype strain 3189. These results indicate that a detoxified LOS-protein conjugate is a candidate vaccine for otitis media and pneumonitis caused by NTHi.

Acute otitis media (OM) and OM with effusion are common childhood diseases (28, 48). It has been estimated that ~75% of children will have had one episode of OM by 3 years of age and ~25% will have had at least three episodes. The highest incidence of OM occurs between 7 and 12 months of age (28, 48), and 10% of these patients suffer persistent middle ear inflammation and mild to moderate hearing loss (15). The annual cost of the medical and surgical treatment of OM in the United States is between $3 billion and $4 billion (3). Furthermore, inappropriate antibiotic treatment of OM encourages the emergence of multidrug-resistant strains of bacteria (3), underscoring the importance of preventing OM in addition to treating it.

Nontypeable *Haemophilus influenzae* (NTHi) is a leading cause of OM and respiratory infections (4, 26, 35). *H. influenzae* conjugate vaccines currently in widespread use are effective only against type b capsulated strains and do not confer protective immunity against infection with NTHi strains. There is no clear understanding of the protective antigens, and there have been no clinical studies to identify an in vitro correlate of protective immunity to NTHi. Some investigators suggested that serum bactericidal antibody is associated with protection from NTHi infection (12, 42). Efforts to develop vaccines against NTHi strains that cause OM have evaluated surface antigens such as outer membrane proteins, pili, and fimbriae (2, 5, 9, 16, 34, 44). One antigen is the P6 protein, because it appears to be antigenically conserved in *H. influenzae* strains and antibodies against P6 are bactericidal (34). Experimental results with this antigen have been variable. Mucosal immunization of rats with P6 enhanced bacterial clearance following NTHi pulmonary challenge (30); however, systemic immunization did not protect chinchilla against NTHi OM (18).

Lipooligosaccharide (LOS), a major surface antigen of NTHi, may be important in the pathogenesis of OM (10) and a protective antigen. Barenkamp and Bodor (1a) reported that human antibodies to NTHi LOS were bactericidal in vitro. McGehee et al. (33) showed that a mouse monoclonal antibody to NTHi LOS was opsonic and that it enhanced bacterial clearance in a murine pulmonary challenge model.

H. influenzae LOSs contain an oligosaccharide linked to lipid A without an O-specific polysaccharide (37, 38). One LOS structure from a deep-rough mutant strain of *H. influenzae* type b had a 2-keto-3-deoxyoctulosonic acid (KDO) linked to lipid A (24). Two primary oligosaccharide structures of LOSs from NTHi 2019 (37) and *H. influenzae* type b strain A2 (38) have been characterized. NTHi LOSs are antigenically heterogeneous when studied by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunologic methods (6, 36). To date, at least five major serotypes of NTHi LOSs have been identified (6). In this study, we used NTHi 9274 LOS (type III) as a source of LOS. LOS from this strain does not have a terminal lacto-*N*-neotetraose, a glycolipid found in a variety of human cells (32), and this LOS induces bactericidal antibodies in mice (22). The LOS was treated with anhydrous hydrazine and coupled to tetanus toxoid (TT) or

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high-molecular-weight proteins (HMP) from NTHi as conjugates. The antigenicity and immunogenicity of these conjugates were evaluated.

MATERIALS AND METHODS

Bacterial growth and LOS purification. NTHi 9274, isolated from middle ear fluid removed from a patient with OM, was provided by M. A. Apicella, University of Iowa. The strain was grown on chocolate agar at 37°C under 5% CO_2 for 8 h and transferred to 200 ml of 3% brain heart infusion medium (Difco Laboratories, Detroit, Mich.) containing NAD (5 μg/ml) and hemin (2 μg/ml) (Sigma Chemical Co., St. Louis, Mo.) in a 500-ml bottle. The bottle was incubated at 150 rpm in an incubator shaker (model G-25; New Brunswick Scientific, Co., Edison, N.J.) at 37°C overnight. The culture was transferred to five 2.8-liter baffled Fernbach flasks, each of which contained 1.4 liters of the same medium. The flasks were shaken at 140 rpm and maintained at 37°C for 24 h. The culture was centrifuged at 15,000 × *g* at 4°C for 30 min to separate the cells and the supernatant. LOS was purified from cells by a modified phenol-water extraction (21) and from the culture supernatant by gel filtration (19). The protein and nucleic acid contents of both purified LOSs were less than 1% (45, 52).

Detoxification of LOS. Anhydrous hydrazine treatment of lipopolysaccharides (LPSs) under mild condition removes esterified fatty acids from lipid A (23). LOS (160 mg), each lot, was dried over P_2O_5 for 3 days, suspended in 16 ml of anhydrous hydrazine (Sigma), and incubated at 37°C for 2 h with mixing every 15 min. This suspension was cooled on ice and added dropwise to cold acetone in an ice bath until a precipitate formed (≥90% acetone). The mixture was centrifuged at 5,000 × *g* at 5°C for 30 min. The pellet was washed twice with cold acetone and dissolved in pyrogen-free water at a final concentration of 20 mg/ml. The reaction mixture was ultracentrifuged at 150,000 × *g* at 5°C for 3 h. The supernatant was freeze-dried and passed through a column (1.6 by 90 cm) of Sephadex G-50 (Pharmacia LKB Biotechnology, Uppsala, Sweden), eluted with 25 mM ammonium acetate, and monitored with a differential refractometer (R-400; Waters, Milford, Mass.). The eluate was assayed for carbohydrate by the phenol-sulfuric acid method (11). The carbohydrate-containing fractions were pooled, freeze-dried three times to remove the salt, and designated dLOS. The yields of the dLOS from three lots ranged from 48 to 55% by weight. For all material and reagent preparations, glassware was baked and pyrogen-free water was used.

Derivatization of dLOS. Adipic acid dihydrazide (ADH) (Aldrich Chemical Co., Milwaukee, Wis.) was bound to the carboxyl group of the KDO moiety of the dLOS to form adipic hydrazide (AH)-dLOS derivatives with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide HCl (EDC) and *N*-hydroxysulfosuccinimide (Pierce) (20, 46). dLOS (70 mg) was dissolved in 7 ml of 345 mM ADH (the molar ratio of ADH to LOS is ~100:1 based on an estimated 3,000 M_r for dLOS) (14, 24). *N*-Hydroxysulfosuccinimide was added to a concentration of 8 mM, the pH was adjusted to 4.8 with 1 M HCl, and EDC was added to a concentration of 0.1 M. The reaction mixture was stirred and maintained at pH 4.8 ± 0.2 with 1 M HCl for 3 h at room temperature. It was adjusted to pH 7.0 with NaOH and passed through the G-50 column as described above. The eluate was assayed for carbohydrate and for AH by a modification of a previously described method (27) by measuring the A_{490} of AH groups. The peaks containing both carbohydrate and AH were pooled, freeze-dried three times to remove the salt, and designated AH-dLOS. AH-dLOS was measured for its composition with dLOS and ADH as standards (11, 27).

Conjugation of AH-dLOS to proteins. TT was obtained from Connaught Laboratories, Inc., Swiftwater, Pa. HMP was purified from NTHi 12 (1). AH-dLOS was coupled to carboxyl groups on TT or HMP at pH 5.6 with EDC. AH-dLOS (20 mg) was dissolved in 2 ml of water and mixed with 10 mg of TT (5.9 mg/ml) or with 8 mg of HMP (4 mg/ml). The molar ratio of AH-dLOS to both TT (M_r, 150,000) and HMP (M_r, 120,000) was ~100:1. The pH was adjusted to 5.6 with 0.1 M HCl, and EDC was added to a concentration of 0.1 M. The reaction mixture was stirred for 1 to 3 h at room temperature; the pH was maintained at 5.6 ± 0.2 with 0.1 M HCl. The reaction mixture was adjusted to pH 7.0, centrifuged at 1,000 × *g* for 10 min, and passed through a column (1.6 by 90 cm) of Sephacryl S-300 in 0.9% NaCl. Peaks that contained both protein and carbohydrate were pooled and designated dLOS-TT or dLOS-HMP. Both conjugates were analyzed for their composition of carbohydrate and protein with dLOS and bovine serum albumin (BSA) as standards (11, 45).

Antigenicity. The antigenicity of dLOS, AH-dLOS, and conjugates was tested by double immunodiffusion and/or enzyme-linked immunosorbent assay (ELISA) (20) with hyperimmune serum to 9274 whole cells and a mouse monoclonal antibody 6245B4 to 9274 LOS (22). The MAb (immunoglobulin M IgM) was assigned a value of 10,000 U/ml by ELISA. For the hyperimmune serum, 10 BALB/c mice were given a total of three intraperitoneal injections at 2-week intervals with about 10^9 bacteria per injection. Blood samples were collected 1 week after the third injection.

Double immunodiffusion was performed in 0.8% agarose in phosphate-buffered saline (PBS; pH 7.4). For ELISA, a 100-μl sample of conjugates at 10 μg/ml (carbohydrate) in PBS or of LOS at 10 μg/ml in PBS containing 10 mM $MgCl_2$ was used as a coating antigen overnight in microplates (Immuno I plate; Dynatech Laboratories, Inc., Alexandria, Va.). The plate was blocked with 150 μl of 1% BSA in PBS for 1 h, 100 μl of diluted mouse serum or monoclonal antibody was added, and the mixture was incubated for 3 h. Alkaline phosphatase-conjugated goat anti-mouse IgG or IgM (Sigma) was added, and the mixture was incubated for 2 h. All steps were performed at room temperature, and 0.9% NaCl containing 0.05% Tween 20 (pH 7.4) was used in five washings between steps. Diluents for sera and phosphatase were 1% BSA and 0.05% Tween 20 in PBS (pH 7.4). After the enzyme substrate was added for 30 min, the reactions were read with a microplate autoreader (EL309; Bio-Tek Instruments) at A_{405}.

Immunogenicity. General-purpose female mice (NIH/Swiss), 5 weeks old (10 mice per group), were given subcutaneous injections with 5 μg of the conjugates (carbohydrate), LOS, dLOS, dLOS plus TT (10 μg), or TT only in 0.2 ml of 0.9% NaCl with or without Ribi-700 adjuvant (containing 50 μg of monophosphoryl lipid A and 50 μg of synthetic trehalose dicorynomycolate) (Ribi ImmunoChem Research, Inc., Hamilton, Mont.). The mice were given a total of three injections at 3-week intervals and bled 14 days after the first injection and 7 days after the second and third injections.

Female New Zealand White rabbits, weighing 2 to 3 kg (two or three per group), were given a total of two subcutaneous and intramuscular injections at 4-week intervals with 50 μg of dLOS or conjugates (carbohydrate) in 1 ml of 0.9% NaCl with or without Ribi-700 adjuvant (containing 250 μg of monophosphoryl lipid A and 250 μg of synthetic trehalose dicorynomycolate) and bled 2 weeks after the first injection and 11 to 14 days after the second injection.

Anti-LOS levels in serum were expressed as ELISA units, with 9274 LOS as a coating antigen and with the hyperimmune serum to NTHi 9274 whole cells as a reference, assigned values of 4,000 and 3,500 U/ml for IgG and IgM, respectively.

The level of TT antibody in serum was measured by ELISA in which TT (5 μg/ml) was used as a coating antigen and a horse anti-TT serum (20 IU/ml) was used as a reference, assigned a value of 320 ELISA U/ml for both IgG and IgM.

The level of HMP antibody in serum was measured by ELISA in which HMP (5 μg/ml) was used as a coating antigen and was expressed as ELISA units on the basis of a reference mouse serum produced by three injections of HMP and assigned values of 2,000 and 10 ELISA U/ml for IgG and IgM, respectively.

Mouse lethal toxicity test. The LOS and dLOS were tested by a lethality assay in mice (13) that was performed by E. Quakyi, U.S. Food Drug Administration, Bethesda, Md.

LAL assay, rabbit pyrogen test, and general safety. The LOS, dLOS, and conjugates were tested by the *Limulus* amebocyte lysate (LAL) assay (25). All reagents were from the Food and Drug Administration. The sensitivity of the LAL assay is 0.09 EU/ml. The pyrogenicity of the conjugates in rabbits was assayed by H. D. Hochstein, Food and Drug Administration. A 25-μg volume (carbohydrate content) of each conjugate, which is assumed to be one human dose (50), was injected intravenously into each rabbit. The conjugates were also assayed for general safety by Hochstein (7).

Bactericidal assay. Rabbit pre- and postimmune sera (after two injections) were inactivated at 56°C for 30 min and tested for bactericidal activity against the homologous strain 9274 and the prototype strain 3198 (type III) (6) by a microbactericidal assay (20). Briefly, twofold dilutions of the sera were made in Dulbecco's PBS containing calcium, magnesium, and 0.1% gelatin (DPBSG) so that 50 μl of sera or diluted sera was present in each well of a sterile 96-well plate. NTHi strains were grown on chocolate plates at 37°C under 5% CO_2 overnight, and three to five colonies were transferred to another plate and incubated for 4.5 h. The bacteria were diluted in DPBSG, and 30 μl of bacterial suspension (about 3×10^3 CFU/ml) was added to each well. Infant-rabbit serum was added (15 μl per well) as a source of complement (Pel-Freez, Brown Deer, Wis.). The plates were incubated at 37°C for 30 min. Then 50 μl of the mixture was removed from each well and spread onto chocolate plates (100 by 15 mm). The plates were incubated at 37°C under 5% CO_2 overnight, and the colonies were counted. Controls included complement, inactive complement, and a positive serum sample. The highest serum dilution causing >50% killing was expressed as the reciprocal bactericidal titer.

Analysis of OS. The oligosaccharide (OS) was isolated from strain 9274 LOS by hydrolysis of the LOS in 1% acetic acid at 100°C for 2 h and purified through the G-50 column with 25 mM ammonium acetate as an eluting buffer. A major OS peak was collected as measured by the phenol-sulfuric acid method (11). The monosaccharide composition of the dephosphorylated OS was determined by high-pressure liquid chromatography (HPLC) as described previously (20).

SDS-PAGE and silver stain. The SDS-PAGE and silver stain were performed as described previously (21, 49).

Statistical analysis. Antibody levels are expressed as the geometric mean (GM) ELISA units or titers (reciprocal) of *n* independent observations ± standard deviation (SD) or range ($n < 4$). Significance was tested with the two-sided *t* test, and *P* values smaller than 0.05 were considered significant.

RESULTS

Characterization of dLOS. The migration of LOS and dLOS on a silver-stained SDS-PAGE gel is showed in Fig. 1. dLOS showed a faster migration, indicating its lower molecular weight than that of LOS. There was no detectable LOS in 10 μg of dLOS (lane 4), although 400-fold more dLOS was loaded

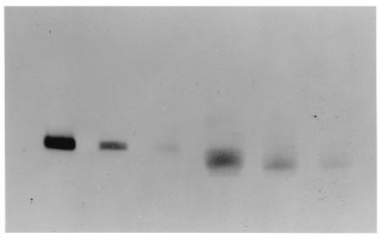

FIG. 1. Silver-stained SDS-PAGE patterns of LOS and dLOS from NTHi 9274. Lanes 1 through 3 contain 100, 50, and 25 ng, respectively, of LOS, and lanes 4 through 6 contain 10, 5, and 2.5 μg, respectively, of dLOS.

on the gel than the amount (25 ng) of LOS (lane 3). This result showed that the residual LOS in dLOS samples was less than 0.25%.

By HPLC analysis, the monosaccharide composition of the OS showed galactose, glucose, heptose, and KDO in a molar ratio of 2.6:3.1:3:1. By the LAL assay, the LOS showed 10,000 EU/μg while the dLOS showed 1 EU/μg (a 10,000-fold reduction). The 50% lethal dose of the dLOS in galactosamine-sensitized mice was 18.3 μg, compared with 0.3 ng of LOS (a 61,000-fold reduction). LOS and dLOS yielded similar precipitation lines in the double-immunodiffusion assay, indicating that dLOS retained the antigenicity of LOS (Fig. 2A).

Characterization of AH-dLOS. The molar ratios of AH to dLOS were 0.47 and 0.55, and the yields, on the basis of carbohydrate content, were 91 and 95% for two lots of AH-dLOS. The AH-dLOS and LOS showed similar precipitation lines in the double-immunodiffusion assay (Fig. 2A).

Characterization of conjugates. The molar ratio of dLOS to proteins in three conjugates ranged from 26:1 to 50:1, and the yield ranged from 11 to 15% on the basis of carbohydrate content (Table 1).

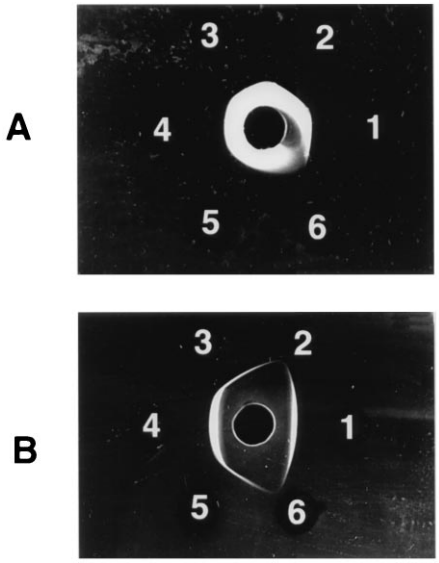

FIG. 2. Double-immunodiffusion assay. The central wells contain mouse monoclonal antibody against NTHi 9274 LOS. (A) Wells: 1, LOS, 2 mg/ml; 2 and 3, AH-dLOS1 and AH-dLOS2, 2 mg/ml; 4 and 5, dLOS1 and dLOS2, 2 mg/ml; 6, TT, 500 μg/ml. (B) Wells: 1, LOS, 2 mg/ml; 2, normal saline; 3, dLOS-TT1, 100 μg/ml; 4, dLOS-TT1, 500 μg/ml; 5, dLOS-TT2, 100 μg/ml; 6, TT, 500 μg/ml.

TABLE 1. Composition, yield, and antigenicity of conjugates used in this study

Conjugate	Amt (μg/ml) of:		Molar ratio of dLOS to protein[a]	Yield[b] (%)	A_{405}[c] (hyperimmune sera/MAb)
	dLOS	Protein			
dLOS-TT1	100	190	26	15	1.56/1.49
dLOS-TT2	90	90	50	11	1.50/1.38
dLOS-HMP	235	274	34	15	1.53/1.45

[a] The ratio is expressed as moles of dLOS per mole of protein with molecular weights of 3,000 for dLOS (14, 24), 150,000 for TT (20), and 120,000 for HMP (2).

[b] Based on the starting amounts of dLOS and the dLOS contained in the conjugates as measured by the phenol-sulfuric acid method (11).

[c] The antigenicity of conjugates was expressed as ELISA reactivity at A_{405} when the conjugates were used as coating antigens (10 μg/ml) and the mouse hyperimmune sera (1/500) and monoclonal antibody (MAb) 6245B4 (1/1,000) were used as binding antibodies. LOS (10 μg/ml) also showed A_{405} values of 1.02 and 1.10 under the same conditions.

By the thermal induction test in rabbits, the dLOS-TT was not pyrogenic when injected intravenously with 25 μg of conjugate per rabbit, or 8 to 9 μg of carbohydrate content per kg of rabbit body weight. By the LAL assay, the dLOS-TT has a carbohydrate content of 0.05 EU/μg. The conjugates met the requirements of general safety tests in guinea pigs at 20 human doses and in mice at 2 human doses (25 μg of dLOS per dose).

The dLOS-TT and the LOS formed identical precipitation lines in the double-immunodiffusion assay (Fig. 2B). Both showed comparable binding to the LOS monoclonal antibody and hyperimmunosera by ELISA (Table 1). The dLOS-HMP showed similar results to dLOS-TT (Table 1).

LOS antibodies in mice. dLOS or a mixture of dLOS and TT did not elicit LOS antibodies in mice (Table 2).

dLOS-TT conjugates elicited low levels of IgG after the first injection (not significant); these levels rose 28- to 162-fold after the second and third injections ($P < 0.01$). dLOS-HMP elicited significant levels of IgG after the first injection; these also rose after the second and third injections (104- to 486-fold above the prevaccination level; $P < 0.01$). After the third injection, dLOS-HMP elicited higher levels of IgG than did dLOS-TT1 ($P < 0.01$) or dLOS-TT2 ($P < 0.05$). LOS alone elicited low levels of IgG after the first injection; these levels rose after the second and third injections (25- to 84-fold above the prevaccination levels; $P < 0.01$). dLOS-TT2 and dLOS-HMP elicited higher levels of IgG anti-LOS than did the LOS alone.

Combination of the three conjugates with the Ribi adjuvant significantly enhanced their immunogenicity: one dose elicited IgG levels comparable to or higher than those after two doses of the conjugates alone, and there was about a fivefold rise in the IgG after three injections ($P < 0.01$).

Conjugates elicited low to medium levels of IgM after each injection. LOS elicited high levels of IgM after the first injection, and these rose after the second and third injections ($P < 0.01$). The Ribi adjuvant enhanced the levels of IgM in the conjugate groups.

TT antibodies in mice. dLOS did not elicit TT antibodies (Table 3). Both dLOS-TTs elicited IgG anti-TT after the first injection; the level of anti-TT significantly rose after the second and third injections ($P < 0.01$). TT alone or a mixture of TT and dLOS showed better IgG responses than did the conjugates after two or three injections ($P < 0.01$ or $P < 0.05$). The Ribi adjuvant enhanced the levels of IgG in all the conjugate groups.

The conjugates, TT, or the mixture of TT and dLOS elicited low levels of IgM after each injection. The Ribi adjuvant enhanced the levels of IgM in the conjugate groups.

TABLE 2. Murine antibody response to NTHi 9274 LOS elicited by conjugates

Immunogen[a]	Injection no.	GM (± SD range) ELISA units[b] for: IgG	IgM
dLOS	1	5 (3–9)	5 (3–8)
	2	6 (3–10)	3 (1–5)
	3	4 (3–7)	2 (1–3)
dLOS-TT1	1	8 (5–13)$^{+}$	14 (8–25)
	2	140 (55–352)$^{++}$	42 (7–237)
	3	217 (91–516)	52 (11–251)
dLOS-TT2	1	10 (3–36)$^{+}$	17 (4–84)
	2	217 (47–1,007)$^{++}$	42 (7–237)
	3	810 (229–2,890)	42 (24–74)
dLOS-TT2 + adjuvant	1	270 (62–1,168)*	470 (266–828)
	2	1,257 (313–5,045)**	101 (64–160)
	3	4,698 (2,664–8,284)	81 (39–169)
dLOS-HMP	1	72 (45–115)$^{+}$	11 (7–18)
	2	522 (296–920)$^{++}$	101 (64–160)
	3	2,430 (1,168–5,055)	14 (8–25)
dLOS-HMP + adjuvant	1	1,403 (780–2,524)$^{+}$	243 (91–516)
	2	7,290 (2,252–23,593)$^{++}$	421 (234–757)
	3	11,006 (6,233–19,435)	19 (11–33)
dLOS + TT	1	4 (3–7)	2 (2–4)
	2	4 (3–5)	3 (1–6)
	3	4 (3–7)	3 (1–11)
LOS	1	65 (38–110)*	195 (123–310)
	2	125 (60–263)**	338 (161–709)
	3	419 (238–739)	470 (186–1,186)

[a] Ten mice for each group were given a total of three subcutaneous injections at 3-week intervals with 5 μg of LOS, dLOS, conjugates, or HMP or with 10 μg of TT. Blood samples were collected 2 weeks after the first injection and 1 week after the second and third injections.

[b] The ELISA units were based on a reference serum against NTHi 9274, and the NTHi 9274 LOS was used as a coating antigen. Preimmune sera contained 5 (range, 3 to 9) U of IgG and 3 (range, 1 to 6) U of IgM. Symbols: * versus **, $P < 0.05$; $^{+}$ versus $^{++}$, $P < 0.01$.

TABLE 3. Murine antibody response to TT elicited by dLOS-TT conjugates

Immunogen[a]	Injection no.	GM (± SD range) ELISA units[b] for: IgG	IgM
dLOS	1	<1	<3
	2	<1	<3
	3	<1	<3
dLOS-TT1	1	30 (14–62)*	16 (9–27)
	2	112 (29–433)**	37 (12–116)
	3	419 (238–739)	12 (8–20)
dLOS-TT2	1	8 (3–25)$^{+}$	12 (8–20)
	2	90 (17–463)$^{++}$	19 (8–49)
	3	522 (296–920)	4 (3–7)
dLOS-TT2 + adjuvant	1	72 (13–397)$^{+}$	30 (14–50)
	2	1,257 (713–2,217)$^{++}$	72 (45–115)
	3	1,257 (713–2,217)	112 (71–178)
dLOS + TT	1	37 (24–59)$^{+}$	4 (3–7)
	2	1,257 (713–2,217)$^{++}$	12 (8–20)
	3	2,430 (863–6,846)	19 (8–49)
TT	1	24 (15–38)$^{+}$	4 (3–7)
	2	650 (409–1,033)$^{++}$	12 (5–30)
	3	1,951 (505–7,528)	19 (4–93)

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

[b] The ELISA units were based on a reference serum against TT, and TT was used as a coating antigen. Symbols: * versus **, $P < 0.05$; $^{+}$ versus $^{++}$, $P < 0.01$.

HMP antibodies in mice. dLOS alone did not elicit HMP antibodies (Table 4). For IgG, dLOS-HMP elicited low levels after the first injection, and these levels rose significantly after the second and third injections ($P < 0.01$). HMP alone showed similar results to dLOS-HMP, and there was no significant difference in IgG levels between the two groups after three injections ($P > 0.05$). The Ribi adjuvant significantly enhanced the levels of IgG in the conjugate group ($P < 0.01$).

dLOS-HMP or HMP alone elicited low levels of IgM after each injection. The Ribi adjuvant enhanced the levels of IgM in dLOS-HMP, and these rose significantly after each injection ($P < 0.01$).

LOS antibodies in rabbits. dLOS alone did not elicit antibodies in rabbits (Table 5). For IgG, dLOS-TT elicited a significant rise in the level of antibodies after the first and second injections (39- to 168-fold above the prevaccination sera; $P < 0.01$). dLOS-HMP showed similar results to those of dLOS-TT (27- to 243-fold above the prevaccination sera). The Ribi adjuvant enhanced the IgG in dLOS-TT ($P < 0.05$) but not in dLOS-HMP after the second injection.

Both conjugates alone and conjugates with the Ribi adjuvant elicited low levels of IgM after each injection.

TT antibodies in rabbits. dLOS did not elicit TT antibodies (Table 6). dLOS-TT elicited IgG anti-TT after the first injection, and these levels rose significantly after the second injection. The Ribi adjuvant enhanced the levels of IgG in the conjugate by threefold after the second injection.

Both conjugate and conjugate with the Ribi adjuvant elicited low levels of IgM after each injection.

HMP antibodies in rabbits. dLOS did not elicit HMP antibodies (Table 7). dLOS-HMP elicited IgG anti-HMP after the

TABLE 4. Murine antibody response to HMP of NTHi elicited by dLOS-HMP conjugates

Immunogen[a]	Injection no.	GM (± SD range) ELISA units[b] for: IgG	IgM
dLOS	1	<1	<3
	2	<1	<3
	3	<1	<3
dLOS-HMP	1	2 (1–3)$^{+}$	6 (3–12)
	2	243 (117–506)$^{++}$	12 (8–20)
	3	1,756 (732–4,134)	8 (3–19)
dLOS-HMP + adjuvant	1	243 (123–490)$^{+}$	5 (3–10)
	2	2,187 (1,096–4,365)$^{++}$	52 (29–93)
	3	>6,561	130 (74–229)
HMP	1	1	3
	2	729 (205–2,592)	5 (3–10)
	3	1,516 (860–2,674)	7 (4–12)

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

[b] The ELISA units were based on a reference serum against HMP, and HMP was used as a coating antigen. Symbols: $^{+}$ versus $^{++}$, $P < 0.01$.

TABLE 5. Rabbit antibody response to NTHi 9274 LOS elicited by conjugates

Immunogen[a]	Injection no.	GM (range) ELISA units[b] for:	
		IgG	IgM
dLOS	1	1	<1
	2	1	<1
dLOS-TT	1	39 (27–81)*	9
	2	168 (81–243)**	9
dLOS-TT + adjuvant	1	27	5 (3–9)
	2	421 (243–729)$^{++}$	16 (9–27)
dLOS-HMP	1	27	3
	2	243	9
dLOS-HMP + adjuvant	1	81	9
	2	243	9

[a] Two or three rabbits for each group were subcutaneously immunized on days 0 and 28 with 50 μg of dLOS, conjugates, or conjugates with Ribi adjuvant. Blood samples were collected on days 0, 14, and 38 to 42.
[b] See Table 2, footnote *b*. Preimmune sera contained 1 U for IgG and <1 U for IgM. Symbols: * versus **, $P < 0.05$; * versus $^{++}$, $P < 0.01$.

first injection, and these levels rose significantly after the second injection. The Ribi adjuvant enhanced the levels of IgG elicited by the conjugate by about threefold after the second injection.

Both conjugate and conjugate plus the Ribi adjuvant elicited low levels of IgM after each injection.

Bactericidal activity of rabbit antisera. Prevaccination sera or dLOS antiserum showed no or low levels of bactericidal activity against the homologous strain 9274 (Fig. 3). The dLOS-TT antisera showed bactericidal activity at mean titers of 1:51 (without adjuvant) and 1:64 (with adjuvant), whereas the dLOS-HMP antisera showed activity at a mean titer of 1:64 for both groups. There was a correlation between LOS IgG antibody levels (ELISA) and the bactericidal titers ($r = 0.81$; $P < 0.01$).

There was no bactericidal activity in prevaccination sera against prototype strain 3198 (type III). Both conjugate antisera elicited bactericidal activity against strain 3198, with titers ranging from 1:2 to 1:16.

DISCUSSION

Two approaches for detoxification of LPS or LOS have been used to obtain clinically acceptable polysaccharide or OS from LPS or LOS. Mild-acid treatment of LPS cleaves the lipid A portion from the LPS molecule at the KDO-glucosamine linkage (53); mild-alkali treatment of LPS removes ester-linked fatty acids while preserving amide-linked fatty acids of lipid A (41). An OS/CRM_{197} conjugate, in which the OS was prepared by treatment of NTHi LOS with acetic acid, showed poor immunogenicity (17). Therefore, we used hydrazine, an organic base, for detoxification of LOS (23, 29). Hydrazine treatment of NTHi LOS resulted in a 10,000-fold reduction in the level of "endotoxin," which is at clinically acceptable levels (54). dLOS-TT passed the general safety requirements and was not pyrogenic in rabbits when injected at 8 to 9 μg/kg of rabbit body weight. By comparison, the World Health Organization specification for *H. influenzae* type b conjugate vaccines is to be nonpyrogenic at 1 μg/kg of rabbit body weight (54).

dLOS retained antigenic determinants but was not immunogenic in mice and rabbits. dLOS become immunogenic when it was coupled to proteins by a selective mild conjugation method. The method is simple and efficient and may be applicable to other partially deacylated LPSs or LOSs bearing KDO residues. The resulting conjugates with two protein carriers induced significant IgG antibody responses to LOS in both mice and rabbits. The immunogenicity of the conjugate in both animals was enhanced by using Ribi adjuvant, consistent with our previous study (20, 40).

TABLE 6. Rabbit antibody response to TT elicited by dLOS-TT conjugates

Immunogen[a]	Injection no.	GM (range) ELISA units[b] for:	
		IgG	IgM
dLOS	1	<3	1
	2	<3	1
dLOS-TT	1	19 (9–27)	9
	2	729	13 (9–27)
dLOS-TT + adjuvant	1	16 (9–27)	3
	2	2,187	5 (3–9)

[a] See Table 5, footnote *a*.
[b] See Table 3, footnote *b*.

TABLE 7. Rabbit antibody response to HMP elicited by dLOS-HMP conjugates

Immunogen[a]	Injection no.	GM (range) ELISA units[b] for:	
		IgG	IgM
dLOS	1	<3	1
	2	<3	1
dLOS-HMP	1	16 (9–27)$^{+}$	3
	2	421 (243–729)$^{++}$	3
dLOS-HMP + adjuvant	1	27	3
	2	1,263 (729–2,187)$^{+++}$	3

[a] See Table 5, footnote *a*.
[b] See Table 4, footnote *b*. Symbols: $^{+}$ versus $^{++}$, $P < 0.01$; $^{++}$ versus $^{+++}$, $P = 0.0519$.

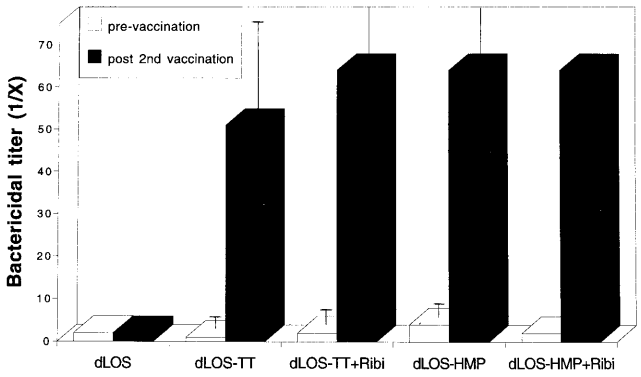

FIG. 3. Bactericidal activity of conjugate-induced antisera against NTHi 9274. Pre- and postvaccination rabbit sera after two injections of dLOS, conjugates, and conjugates with Ribi adjuvant were tested. Each group contained one to three rabbits. The bactericidal titers were given as the serum dilution causing >50% killing of the bacteria and expressed as the geometric mean and standard deviation for each group. The bactericidal titer for the hyperimmune sera was 1:1,600.

We used HMP (2), an adhesion molecule of the outer membrane proteins from NTHi, to produce the dLOS conjugate. The preliminary result showed that dLOS-HMP was more immunogenic than dLOS-TT in inducing anti-LOS IgG responses in mice. The dLOS-HMP also elicited significant anti-HMP IgG in both mice and rabbits, which might be useful for inhibiting the adhesion of NTHi in vivo (1, 47).

Conjugate-induced rabbit antisera but not mice antisera showed bactericidal activity against the homologous strain 9274 and strain 3198 (type III LOS) (6). There was a correlation between LOS IgG antibody levels and the bactericidal titers. These results are consistent with our previous results obtained with meningicoccal LOS-derived OS-TT conjugates (20). Many factors may result in the lack of bactericidal antibodies generated by the conjugates in mice; these include immunization routes, antibody titers and affinities, animal species, and sources of complement.

Bactericidal LPS or polysaccharide antibodies in serum confer protective immunity to many human pathogens (39) including *H. influenzae* type b, *Neisseria meningitidis*, *Vibrio cholerae*, and, as recently found, *Shigella sonnei* (8). In the case of OM, vaccination of children older than 2 years with pneumococcal polysaccharides (31) or administration of hyperimmune human immune globulin (43), which contains antibodies to pneumococcal polysaccharide, to otitis-prone children resulted in a decrease in the incidence of pneumococcal OM. Children with NTHi OM lack bactericidal antibodies before their infection and develop strain-specific bactericidal antibody following infection (12). The bactericidal titers of the convalescent-phase sera from children who recovered from acute NTHi OM were 1:8 to 1:32 (1a). We suggest that dLOS-TT- or dLOS-HMP-induced bactericidal antibodies (IgG) can transude to mucosal surfaces (51) of nasopharynges, probably eliminating NTHi OM and respiratory diseases caused by NTHi. For evaluation of the dLOS-protein conjugate as a potential vaccine for human use, further studies are needed to investigate the conjugate antisera in terms of opsonization, endotoxin neutralization, and protection in animal models. Multivalent conjugates derived from a few types of NTHi LOSs will be necessary to cover most, if not all, NTHi disease strains.

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