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Quantitation and Biological Properties of Released and Cell-Bound Lipooligosaccharides from Nontypeable *Haemophilus influenzae*

XIN-XING GU,[1]* CHAO-MING TSAI,[2] MICHAEL A. APICELLA,[3] AND DAVID J. LIM[1]

Laboratory of Cellular Biology, National Institute on Deafness and Other Communication Disorders,[1] and Center for Biologics Evaluation and Research, U.S. Food and Drug Administration,[2] Bethesda, Maryland, and Department of Microbiology, University of Iowa, Iowa City, Iowa[3]

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Nontypeable *Haemophilus influenzae* (NTHi) is a major pathogen causing otitis media in children. NTHi releases lipooligosaccharide (LOS) as outer membrane fragments during its growth. The release of LOS may play an important role in the pathogenicity of otitis media caused by this organism. The amounts of LOS in bacterial cells and growth media for five NTHi strains were determined by quantitative silver staining after sodium dodecyl sulfate-polyacrylamide gel electrophoresis. These strains were estimated to have 1.6×10^6 to 4.8×10^6 LOS molecules per bacterium. During a 3-day growth period, these NTHi strains released variable but significant amounts of LOS into the growth medium. Cells started to release detectable amounts of LOS into the medium at 2 to 5 h and continued to do so for up to 48 or 72 h. The concentrations of LOS in the culture supernatants released by these five strains were 10 to 55 μg/ml at 24 h and 40 to 100 μg/ml at 72 h, which was 34 to 189% of the cell-bound LOS concentration. The biological properties of released and cell-bound LOSs from two representative strains were compared. Released LOS showed an approximately 10-fold increase in inducing human monocytes to produce tumor necrosis factor alpha, interleukin 1β, and interleukin 6, a 13- to 28-fold increase in mouse lethal toxicity, and a 16- to 37-fold increase in the clotting of *Limulus* amebocyte lysate. These results suggested that released LOS or its inflammatory mediators play a more important role than the LOS in bacteria in the pathogenicity of otitis media caused by this organism.

Nontypeable (or noncapsulated) *Haemophilus influenzae* (NTHi) is a major cause of otitis media in children (6). However, the exact pathogenic mechanisms by which this organism causes otitis media have not yet been established. Endotoxin or lipopolysaccharide (LPS) in this gram-negative bacterium has been recovered from the middle ears of patients with both acute and chronic otitis media with effusion (12, 32, 33). It has been suggested that endotoxin is responsible for inner ear pathology in cases of acute and chronic otitis media with effusion (28, 31). Endotoxin is also thought to be cytotoxic to ciliated epithelial cells (14, 26) and to be one of the major factors that cause chronic middle ear effusion even when bacteria are no longer alive (11, 13, 38, 39, 47).

Many gram-negative bacteria can release LPS-containing outer membrane fragments (or free LPS) during growth (2–4, 8, 10, 17, 21, 25, 29, 37, 45, 50), or it can be released by antibiotic and other treatments (16, 34, 35, 43). Unlike LPSs from enterobacteria, NTHi LPS contains only an oligosaccharide linked to lipid A without O-polysaccharide chains; therefore, the term lipooligosaccharide (LOS) has also been used for NTHi LPS (19). LOS released by NTHi may access areas of tissues and persist in these tissues even after the organism is eliminated by antibiotic treatment (12, 32). Therefore, released LOS may become more dominant than bacteria-bound LOS in inducing macrophages and other target cells to produce inflammatory mediators, such as tumor necrosis factor (TNF), interleukin 1 (IL-1), and other cytokines, during the course of NTHi otitis media (9, 15, 36), while the exact pathogenic mechanism of NTHi-induced otitis media has not yet been fully elucidated. These observations prompted us to examine the release of LOS from NTHi strains and the biological properties of released LOS. We hypothesize that released LOS is a major factor that causes an inflammatory response in middle ear tissue and leads to chronic NTHi otitis media with effusion.

In this study, we have demonstrated that LOS is released from five NTHi strains during growth. The amounts of released and cell-bound LOSs from these strains have been quantified. We have also analyzed the biological activities of released LOS, cell-bound LOS, and purified LOS.

MATERIALS AND METHODS

Bacteria. NTHi strains 9274, 6491, 5756, 2627, and 2019 were from one of our collections (M.A.A.). All of these strains were isolated from the middle ear fluids of patients with otitis media, except strain 2019, which was isolated from a patient with chronic bronchitis.

Growth conditions and sample collection. Strains were grown on chocolate agar at 37°C and 5% CO_2 for 16 h and then transferred to 1.5 liters of 3% brain heart infusion medium (Difco Laboratories, Detroit, Mich.) plus NAD (5 μg/ml) and hemin (2 μg/ml) (Sigma Chemical Co., St. Louis, Mo.) in a 2.8-liter baffled Fernbach flask. Flasks were kept at 150 rpm in an incubator shaker (model G-25; New Brunswick Scientific Co., Edison, N.J.) at 37°C for 3 days. NAD and hemin were also added at 24 and 48 h at the same concentrations of 5 and 2 μg/ml, respectively.

Samples (50 ml) were taken from cultures at 2, 5, 7, 10, 24, 48, and 72 h. The optical densities of bacteria were measured at 600 nm, and viable counts as CFU were obtained by plating samples on chocolate plates and counting colonies on the next day.

These samples were separated into cells and supernatants by centrifugation at $10,000 \times g$ for 20 min. The LOS in cell pellets was considered to be cell-bound LOS, while the LOS in culture supernatants was defined as released LOS (or free LOS). Cell pellets were reconstituted with medium to the original volume. The supernatants with low LOS contents (2 to 10 h) were ultracentrifuged at 150,000 $\times g$ for 3 h, and LOS-containing pellets were resuspended in a small volume of medium. Each strain was grown twice, and samples were collected under the same conditions as described above.

Quantitation of LOS by densitometry after electrophoresis and silver stain-

* Corresponding author. Mailing address: Laboratory of Cellular Biology, NIDCD, NIH, Building 29, Room 402, 8800 Rockville Pike, Bethesda, MD 20892. Phone: (301) 496-2154. Fax: (301) 402-2776.

ing. Cell-bound LOS in cell pellets and released LOS in culture supernatants were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 15% Laemmli gel (30) and then silver stained (49). The LOS standard was prepared from cells by phenol-water extraction (53). For each assay, 25, 50, 100, and 150 (or 200) ng of LOS standard and duplicated samples were mixed with SDS-PAGE digestion buffer, boiled at 100°C for 10 min, and then loaded on an SDS-PAGE gel. For cell pellets, each boiled solution was incubated with 100 μg of proteinase K (final concentration, 0.5 mg/ml) (Sigma) at 60°C for 2 h before being loaded (23). Suitable sample dilution and loading volume were adjusted after initial SDS-PAGE and silver staining to reach the linear range of the LOS standard curve. Silver-stained LOS bands were scanned with a laser densitometer (model 2202; Pharmacia LKB Biotechnology, Inc., Piscataway, N.J.), and the amounts of LOS were calculated on the basis of the standard curve (48). The sensitivity in detecting LOS is about 10 ng or 0.5 to 1 μg of LOS per ml. Each sample was examined in duplicate for each SDS-PAGE, silver staining, and scanning densitometry assay. LOS data are means obtained from six to eight tests.

Estimation of LOS molecules per bacterium. On the basis of cell-bound LOS concentrations and bacterial viability counts at the logarithm growth periods of 5, 7, and 10 h, the number of LOS molecules per bacterium was calculated from Avogadro's number ($n = 6.022 \times 10^{23}$) by using a molecular weight of 4,000 for LOS, which is based on the known structures of oligosaccharides and lipid A from *H. influenzae* LOSs (19, 22).

DNA analysis. To estimate cell lysis during bacterial growth, the DNA contents of culture supernatants and whole-culture media were measured by the method of Burton (7) as modified by Giles and Myers (20) to obtain the percentage of cell lysis.

Purification of LOS. LOS was purified from two NTHi strains by classical phenol-water extraction (53) with modifications. Briefly, overnight-growth cells were treated with 90% phenol (45% of the final concentration) at 68 to 70°C for 15 to 20 min, cooled in ice, and centrifuged. After recovery of the upper aqueous phase, the remainders were reextracted with water. Sodium acetate (5 mg/ml) was added to the combined aqueous phases, and LOS was precipitated with 2 volumes of acetone to reduce phospholipid contamination. Pellets were washed twice with 70% ethanol to reduce trace phenol and then dissolved in water. RNase and DNase were added (50 to 100 μg/ml), and the digestion mixture was kept at 37°C for 3 to 5 h. Proteinase K (0.5 mg/ml) was added, and then the digestion mixture was incubated at 60°C overnight. The digestion mixture was ultracentrifuged twice at 150,000 × *g* for 3 h. Gel-like LOS was dissolved in about 10 volumes of water and lyophilized. The protein and nucleic acid contents of purified LOS were less than 1% (44, 52).

LAL assay. For a *Limulus* amebocyte lysate (LAL) assay and other biological assays, samples of released LOSs from five strains collected at 24 h were passed through a membrane filter (pore size, 0.22 μm) to ensure the absence of any whole bacteria in supernatants. The LOS contents of these supernatants were requantified; there was about a 10% reduction in LOS after filtration. Samples of both released and cell-bound LOSs from five strains were diluted with pyrogen-free water. Equal volumes (100 μl) of samples and LAL were mixed and incubated at 37°C for 1 h. The gelation of lysate at the minimal LOS concentration was determined by inverting the mixture. A firm gel was considered a positive reaction (24). All reagents were from the U.S. Food and Drug Administration, Bethesda, Md. The sensitivity of the LAL assay is 0.09 EU per ml.

Lethal toxicity test. The released, cell-bound, and purified LOSs from strains 9274 and 2019 were further tested by a mouse lethality assay (18). Briefly, female 7-week-old outbred NIH/Swiss mice and inbred BALB/c mice (eight per group) were injected intraperitoneally with 8 mg of D-galactosamine· HCl (Sigma) dissolved in 0.2 ml of pyrogen-free water (about 400 mg/kg). Within 30 min, the animals were given different amounts of experimental preparations in 0.2 ml of water by the intravenous route. Animals receiving D-galactosamine· HCl (0.2 ml intraperitoneally) and water or *Salmonella abortus equi* LPS (Sigma) (0.2 ml intravenously) served as controls. Lethality was observed over 4 days, and the 50% lethal dose (LD_{50}) was calculated.

Cytokine assays. Human peripheral blood monocytes were prepared at the National Institutes of Health Clinical Center (Bethesda, Md.) by countercurrent centrifugal elutriation techniques designed to yield large numbers of purified monocytes suitable for clinical use (1). Monocytes obtained by this method were greater than 90% pure as assessed by histochemical staining.

Monocytes (1.5×10^6/ml) in RPMI medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 50 U of penicillin G per ml, and 50 μg of streptomycin sulfate (GIBCO, Grand Island, N.Y.) per ml were cultured on 96- or 24-well plates (Costar, Cambridge, Mass.) with graded concentrations of different LOS preparations (0.01 ng to 10 μg/ml) for 20 h at 37°C (20a). Aliquots of the culture supernatant were removed, and the levels of TNF alpha (TNF-α), IL-1β, and IL-6 in aliquots were assayed separately by using enzyme-linked immunosorbent assay (ELISA) kits (R & D Systems, Minneapolis, Minn.). For accurate extrapolations, samples were duplicated and diluted to fall within the steep linear portions of standard curves. The results are expressed in picograms per milliliter. The average percent responses are from four individual experiments.

Statistical analysis. Significance was determined with the two-sided *t* test, and *P* values <0.05 were considered significant.

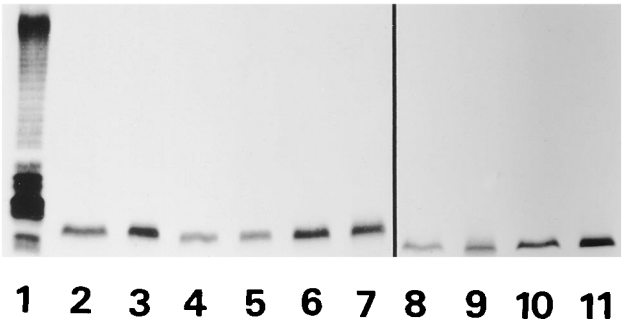

FIG. 1. SDS-PAGE and silver staining patterns of released LOSs (lanes 2, 4, 6, 8, and 10) and cell-bound LOSs (lanes 3, 5, 7, 9, and 11) from NTHi strains 9274 (lanes 2 and 3), 6491 (lanes 4 and 5), 5756 (lanes 6 and 7), 2627 (lanes 8 and 9), and 2019 (lanes 10 and 11) obtained at 24 h. Lane 1, purified smooth LPS from *Salmonella minnesota* (Sigma) used as a control.

RESULTS

Quantitation of released LOS and cell-bound LOS. The released and cell-bound LOSs from each of five NTHi strains appeared to be the same on a silver-stained SDS-PAGE gel (Fig. 1). The amounts of released LOS and cell-bound LOS were quantitated by densitometry after SDS-PAGE and silver staining. Figure 2 shows a silver-stained SDS-PAGE gel of released LOS from strain 5756 for the period from 10 to 72 h and a set of standards for this LOS. From this silver-stained gel (Fig. 2A), LOS peaks were obtained, and peak areas were calculated by densitometry (Fig. 2B). The amounts of released LOS from strain 5756 were quantitated on the basis of a curve derived from LOS standards (Fig. 2C). For strain 5756, the amount of released LOS increased from 2 to 51 ng during 10 to 72 h (Fig. 2C [circles]).

The cell densities and amounts of cell-bound LOS and released LOS during 3 days of growth are summarized in Fig. 3. The amounts of cell-bound LOS reached their maxima at 10 to 24 h when the cell densities were at their peaks (Fig. 3A and B). Cells started to release LOS by 2 to 5 h, and 10 to 55 μg of LOS per ml was detected in supernatants at 24 h (Fig. 3C). The release of LOS from strains 6491 and 5756 ceased at 48 h, while the other three strains continued to release LOS. At 72 h, the amount of released LOS was 40 to 100 μg/ml, which was 34 to 189% of the amount of cell-bound LOS for these five strains. There was an inverse correlation between the levels of released LOS and cell-bound LOS for strains 2627 and 2019. For strain 2627, the amount of released LOS increased from approximately 10 to 100 μg/ml while the amount of cell-bound LOS dropped from approximately 130 to 60 μg/ml during the growth period from 10 to 72 h (Fig. 3B and C). Strain 2019 had a similar pattern for the period from 24 to 72 h.

Among the bacterial viabilities of these five strains, the highest counts were at 10 h (10^{10} CFU/ml), similar to the results of Herriott et al. (22a). However, the viable counts decreased 90% at 24 h. DNA analysis showed that only 1 to 5% of total DNA was present in supernatants at 10 h and that only 10 to 20% was present at 72 h for these five strains. These results suggested that the major part of released LOS was not due to cell lysis. On the basis of the cell-bound LOS concentrations and the bacterial viability counts at 5 to 10 h, the number of LOS molecules per bacterium was calculated to be from 1.6×10^6 to 4.8×10^6, which is similar to that for an *Escherichia coli* strain (37a).

Biological properties of released LOSs. The released and cell-bound LOSs collected at 24 h were tested by an LAL assay. The results are shown in Table 1. The minimal LOS concen-

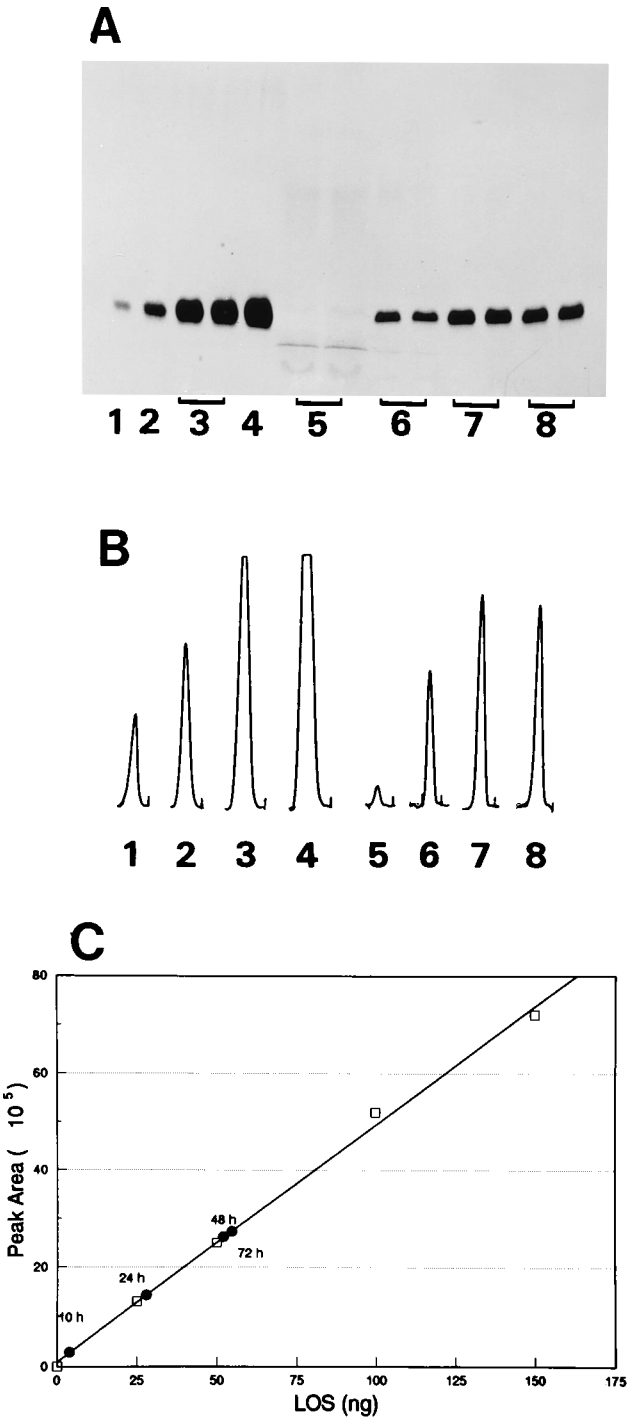

FIG. 2. (A) Silver-stained gel after SDS-PAGE. (B) Densitometric scanning of panel A. Lanes 1 through 4, LOS standards of 25, 50, 100, and 150 ng; lane 3 contains two identical 100-ng samples of LOS. Lanes 5 through 8 (duplicate samples), released LOSs from strain 5756 at 10, 24, 48, and 72 h, respectively. (C) LOS standard curve (squares) and sample LOS concentrations (circles) were based on the peak areas obtained by densitometry in panel B.

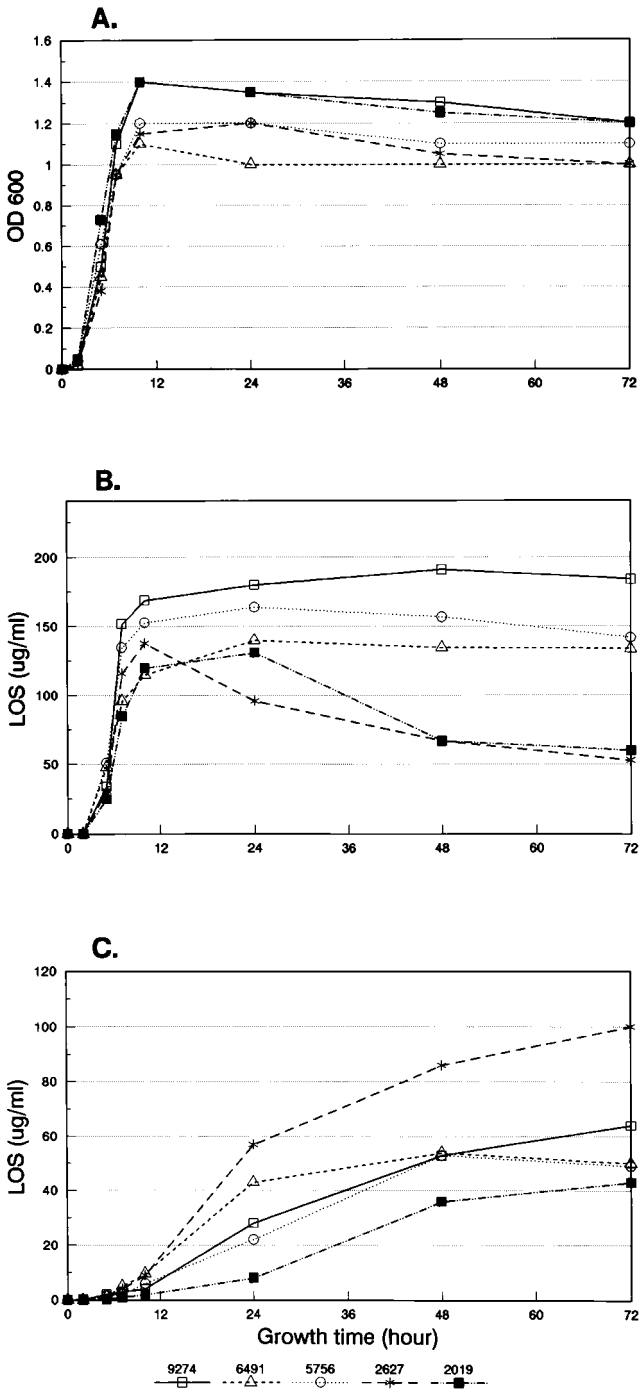

FIG. 3. (A) Cell density curves for five NTHi strains (9274, 6491, 5756, 2627, and 2019) during a growth period of 72 h. The concentrations of cell-bound (B) and released LOSs (C) from the same strains were detected by SDS-PAGE, silver stained, and subsequently quantitated by densitometry during this growth period. OD 600, optical density at 600 nm.

trations required for the gelation of LAL were 1.4 to 2.8 pg/ml for released LOSs from all five strains, while those of cell-bound LOSs were 24 to 82 pg/ml. There is a 16- to 37-fold difference in reactivity between released and cell-bound LOSs in the LAL assay. For two purified LOSs (strains 9274 and 2019), the minimal LOS concentrations required for LAL gelation were 25 and 10 pg/ml, respectively, intermediate values between the LAL activities of released LOSs and cell-bound LOSs.

The toxicities of released, cell-bound, and purified LOSs from strains 9274 and 2019 were tested in mouse models (Table 2). In D-galactosamine-sensitized BALB/c mice, released

TABLE 1. LAL activities of released and cell-bound LOSs from NTHi strains

Strain or species	LOS source[a]	Minimal LOS concn for gelation (pg/ml)[b]
9274	Supernatant	2.8
	Cell	45 (16)
6491	Supernatant	2
	Cell	48 (24)
5756	Supernatant	2.2
	Cell	82 (37)
2627	Supernatant	1.4
	Cell	24 (17)
2019	Supernatant	2
	Cell	66 (33)
E. coli[c]		10

[a] The culture supernatant contains released LOS, while the cell contains cell-bound LOS at the growth time of 24 h.
[b] Numbers in parentheses are fold differences between the LAL activities of cell-bound LOSs and those of released LOSs.
[c] *E. coli* EC-5, the United States standard endotoxin for LAL assays.

and purified LOSs showed similar lethalities, with LD_{50}s of 0.7 to 1.1 ng, while cell-bound LOSs showed the lowest lethalities, with LD_{50}s of 18.1 and 22.1 ng for these two NTHi strains. The amounts of released LOSs from strains 9274 and 2019 necessary for an LD_{50} were 28- and 16-fold less, respectively, than those of cell-bound LOSs from the same strains. Similar results were also obtained with outbred NIH/Swiss mice, with 13- and 16-fold decreases in the LD_{50}s of released LOSs. Ninety percent of mice died within 24 h of LOS challenge, but no outbred or inbred mice died after 48 h.

Since TNF-α, IL-1β, and IL-6 have been implicated as major inflammatory mediators in the pathogenesis of many infectious diseases, including otitis media (9, 15, 27, 36, 40), we studied the effects of released and cell-bound LOSs on cytokine production from monocytes. Human monocytes were stimulated with different LOS preparations from strains 9274 and 2019, and the levels of these three cytokines in culture supernatants were measured at 20 h. The means of four separate experiments with strain 9274 are shown in Fig. 4. The production of all three cytokines was dose dependent. With different LOS preparations, the maximal production of TNF-α (21.3 ng/ml) and IL-1β (6.6 ng/ml) occurred at LOS concentrations of 1 to 10 μg/ml and the maximal production of IL-6 (44.9 ng/ml) occurred at LOS concentrations of 0.1 to 1 μg/ml.

As shown in Fig. 4, 50% of maximal TNF-α and IL-1β production was obtained with 0.7 and 0.9 ng of released LOS per ml, respectively, while 6.9 and 8.5 ng of cell-bound LOS per ml were necessary for 50% of maximal TNF-α and IL-1β production, respectively. For 50% of maximal IL-6 production, 0.09 ng of released LOS per ml, instead of 0.80 ng of cell-bound LOS per ml, was required. The differences (about 10-

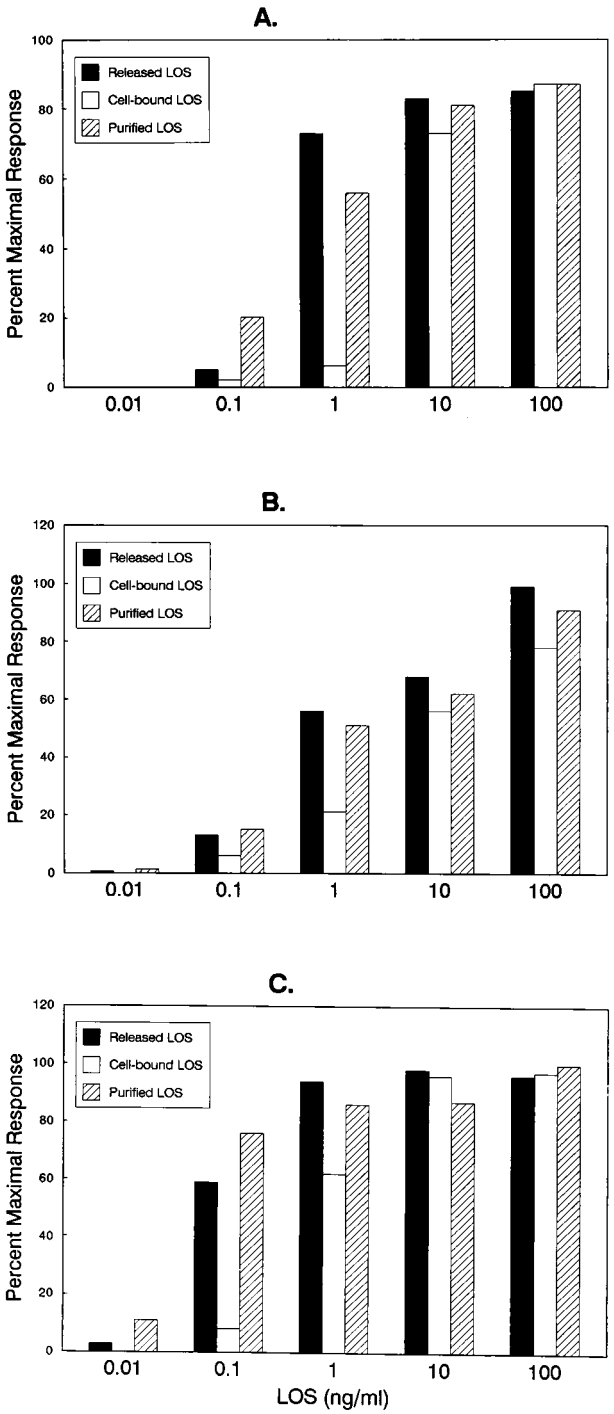

FIG. 4. Induction of TNF-α (A), IL-1β (B), and IL-6 (C) by NTHi LOSs in human monocytes. Monocytes were stimulated by the indicated doses of different LOS preparations. The amounts of TNF-α, IL-1β, and IL-6 were measured by ELISA, and the results are expressed as the percentages of maximal responses. The means of four separate experiments were used to generate the data shown (variation, ±5 to 20%).

TABLE 2. Lethal toxicities of released, cell-bound, and purified LOSs from NTHi strains in D-galactosamine-sensitized mice[a]

Strain	LOS source[b]	LD_{50} (ng)	
		BALB/c	NIH/Swiss
9274	Supernatant	0.8 ± 0.2	1.1 ± 0.2
	Cell	22.1 ± 5.0	14.4 ± 2.8
	Purified	0.7 ± 0.1	0.8 ± 0.2
2019	Supernatant	1.1 ± 0.2	0.8 ± 0.3
	Cell	18.1 ± 3.2	13.0 ± 3.0
	Purified	1.0 ± 0.3	0.9 ± 0.3

[a] Mice, eight per group, were treated intraperitoneally with D-galactosamine (400 mg/kg), and within 30 min, serial dilutions of different LOS preparations were injected intravenously. Dead mice were counted over 4 days, and data are means ± standard deviations of three experiments.
[b] The culture supernatant contains released LOS, the cell contains cell-bound LOS, and purified LOS was extracted by the method of Westphal and Jann (53).

fold) in amounts between released LOS and cell-bound LOS were significant ($P < 0.01$) and occurred at low doses, similar to the concentrations of LOSs found in clinical infections and animal models (13, 32, 33). There were no significant differences between released LOS and purified LOS in stimulating cytokine production ($P > 0.05$). Strain 2019 had results similar to those for strain 9274 (data not shown).

DISCUSSION

Many methods are available for assaying LPS (endotoxin) from gram-negative bacteria. Different methods give various results (24, 35, 37, 41, 42, 51). To date, the most frequently used and convenient assays for the measurement of LPS have been the LAL and 2-keto-3-deoxyoctonic acid (KDO) assays. The KDO assay is not suitable for the detection of LPS in colored culture media or turbid cell suspensions. It cannot be used for *H. influenzae* LOS because the sole KDO in this LOS is substituted at both the fourth and fifth positions. This results in a very low yield of chromophore (41, 41a, 50). The LAL assay is based on exposure of the lipid A portion of the LPS molecule activating the enzyme system in LAL, but some other substances, such as β-glucans, also give positive reactions in this assay (5). In addition, our results show that the same amounts of NTHi LOS in different forms, released versus cell bound, have 16- to 37-fold differences in activity in the LAL assay. In this study, we successfully used quantitative densitometry after SDS-PAGE and silver staining to measure the levels of both released and cell-bound LOSs from NTHi strains. The sensitivity of this method is at least 100-fold higher than that of the KDO assay but is lower than that of the LAL assay.

All five NTHi strains released variable but significant amounts of LOS into the medium during growth. Released LOS is more active in the LAL assay, more toxic in mice, and more potent in inducing cytokine production than is cell-bound LOS. These results are similar to those obtained with free and cell-bound LPSs from enterobacteria (35), although the preparations of cell-free endotoxins and the methods of endotoxin quantitation were different. The reasons for the different activities of released and cell-bound LPSs are rather complex. Released LOS is presumably existing in outer membrane blebs since SDS-PAGE reveals both LOS and outer membrane proteins (data not shown). As in outer membrane blebs, released LOS is much smaller than bacteria, which may give more opportunities to access target cells and to expose lipid A to the receptors of target cells. The low LAL activity and low mouse lethality of cell-bound LOS became as potent as those of released LOS once LOS had been purified from cells (strains 9274 and 2019 [Table 2]). Although there is no direct evidence to support the concept described above, Takayama et al. (46) reported that the monomeric Re-LPS of *E. coli* is much more active than the aggregated Re-LPS preparation in an LAL assay and in the induction of Egr-1 mRNA from peritoneal macrophages.

Our study showed that NTHi strains can release highly active LOS into the culture supernatant whether bacteria are viable or not. Released LOS may be the most biologically active form for the pathogenicity of NTHi otitis media. Continued release of LOS by nonviable NTHi may be a source of continued inflammation, causing persistent middle ear effusion in children. For some patients with chronic otitis media, LOS has been detected in middle ear effusions without positive bacterial cultures (12). It has also been shown in animal experiments that killed bacteria and NTHi-derived LOS can cause otitis media with effusion (11, 13). Further study is needed to investigate the functions of this naturally released LOS in animal models to understand and elucidate the pathogenetic mechanisms of NTHi otitis media.

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