Purification of meningococcal lipo-oligosaccharide by FPLC techniques

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A rapid and efficient method for the preparation of highly pure meningococcal lipo-oligosaccharide (LOS) was developed. This used a Superose 6 column on a FPLC system to purify LOS from phenol–water extracts of cell lysates of Neisseria meningitidis. The purest LOS preparations, with no detectable protein contamination and less than 0.5% (w/w) residual RNA, were obtained when cell lysates had been treated with RNase ONE before phenol extraction and chromatographic separation. Preparations that had received no ribonuclease treatment had 2-3% residual RNA contamination and predigestion of samples with RNase A, which only partially degraded the RNA present in the crude extracts, resulted in LOS samples contaminated with 15-20% residual RNA. The LOS purified from RNase ONE-treated extracts was highly endotoxic, and showed no reduction in antibody binding or specific endotoxin activity compared to unpurified material. Approximately 80% of the LOS applied to the chromatography column was recovered as purified material.

Keywords: Neisseria meningitidis, lipo-oligosaccharide, FPLC, RNA removal

INTRODUCTION

Outer membrane glycolipids, which consist of endotoxic lipid A linked by a core polysaccharide to an antigenically variable saccharide chain, are important in both immunity to and pathology of Gram-negative pathogens (Phillips et al., 1990). The toxicity of these molecules depends on the availability of the lipid A moiety to host receptors, which is largely determined by the variable saccharide chain. Unlike the lipopolysaccharides of the enteric bacteria, which have polysaccharide O-antigen chains of more than 10 sugar units, the highly toxic glycolipids of mucosal pathogens such as the pathogenic species of Neisseria have short saccharide chains of 2–10 sugar units (Campagnari et al., 1990). The term lipo-oligosaccharide (LOS) has been used to distinguish these molecules from LPS (Griffiss et al., 1988; Hitchcock et al., 1986), but this practice has not been uniformly adopted.

The LOS of Neisseria meningitidis, an important cause of meningitis and septicaemia worldwide (Peltola, 1983), is used for strain characterization by defining the meningococcal immunotype (Scholten et al., 1994), is a potential vaccine component (Estabrook et al., 1990) and is the principal cause of potentially fatal purpuric rash and endotoxic shock (Morrison, 1982). Meningococcal strains can express a variety of LOS immunotypes (Gamian et al., 1992; Pavliak et al., 1993), some of which are associated with disseminated disease, and many LOS epitopes are conformational, poorly defined and not stable on purification. A complete understanding of the roles of LOS in immunity and disease requires improved materials and reagents for use in novel techniques such as antibody rescue (Borrebaeck et al., 1992; Brezinschek et al., 1995) and surface resonance analyses (Malmborg et al., 1992).

In the present work, a rapid, efficient method for the purification of meningococcal LOS using chromatography on a Superose 6 column automated with a FPLC system is described. The high resolution, fast elution rate and reproducibility obtained with FPLC reduced the number of fractions to be assayed and decreased processing time. Optimization of the digestion of contaminating RNA improved purity and the use of refractive index to monitor LOS elution (Gamian et al., 1992), allowed immediate identification of relevant fractions. This method produced antigenically active, highly endotoxic LOS of high purity.

MATERIALS AND METHODS

Bacteria and culture conditions. Meningococcal strain K454 (B:15;P1.7,16:L3,7,9) was obtained from the Public Health
overnight (15-16 h) at 37 °C on heated blood (chocolate) agar plates in an atmosphere of 5% (v/v) CO₂. After revival, inocula for liquid cultures in Müller–Hilton broth were prepared by resuspending fresh (overnight) plate growth in Müller–Hilton broth (Difco, 1 ml per plate). These inocula were diluted 1:200 in Müller–Hilton broth and the cultures incubated with shaking (320 r.p.m.) for 8 h at 37 °C.

**Monoclonal antibody.** The monoclonal antibody (mAb) used was 9-2-L379, obtained from Dr W. Zollinger, Walter Reed Army Institute of Research, Washington DC, USA. This antibody had been raised against a strain of *N. meningitidis* with the same immunotype as strain K454 (Scholten *et al.*, 1994). It was used in the form of ascitic fluid, collected from pristane-treated mice after intraperitoneal injection with cells of the hybridoma cell line. Ascitic fluid was stored at −20 °C.

**Extraction of LOS.** Phenol extraction was essentially as described by Johnson & Perry (1976). Cells from Müller–Hilton broth cultures were resuspended in 3 ml 40 mM sodium phosphate buffer, pH 7, containing 5 mM EDTA (g wet wt cells)⁻¹. Lysozyme to a final concentration of 6 mg (g wet wt cells)⁻¹ was added and the cells lysed by overnight incubation at 2–4 °C, with stirring. The lysates were extracted with phenol at 65–70 °C for 15 min and the phases separated by centrifugation at 8750 g for 10 min. After removal of the aqueous phase, the phenol phase was re-extracted with an equal volume of distilled water. The combined aqueous phases were adjusted to 0.5 M NaCl, 10 volumes of 96% (v/v) ethanol were added and the mixture was left overnight at −20 °C (Inzana, 1983). The precipitate was collected by centrifugation, resuspended in distilled water, divided into aliquots suitable for chromatographic separation and freeze-dried.

**RNase digestion.** For samples digested with RNase A, the cell lysate was incubated at 37 °C for 3 h with 150 μg RNase A (Sigma) (g wet wt cells)⁻¹, after Wu *et al.* (1987), before phenol extraction as described above. After resuspension in distilled water, the crude LOS was digested a second time with 150 μg RNase A (Promega) (g wet wt cells)⁻¹ before phenol extraction. The ethanol precipitate obtained after phenol extraction was resuspended in RNase ONE reaction buffer (20 mM Tris/HCl, pH 7-5) and treated with 20 μg RNase ONE (Promega) (g wet wt cells)⁻¹ before phenol extraction. The ethanol precipitate obtained after phenol extraction was resuspended in RNase ONE reaction buffer (20 mM Tris/HCl, pH 7-5) and treated with 20 μg RNase ONE (Promega) (g wet wt cells)⁻¹ before phenol extraction. The ethanol precipitate obtained after phenol extraction was resuspended in RNase ONE reaction buffer (20 mM Tris/HCl, pH 7-5) and treated with 20 μg RNase ONE (Promega) (g wet wt cells)⁻¹ before phenol extraction.

**Column chromatography.** Crude LOS samples were prepared for column chromatography by resuspension in 1-5% (w/v) sodium deoxycholate (SigmaUltra), pH 8-5, to a concentration of 1-2 mg ml⁻¹. For chromatographic separation, a sample of 100 μl was used with 50 mM Tris/HCl, pH 8-5 (SigmaUltra), 5 mM EDTA, 1-5% sodium deoxycholate as the elution buffer (modified from Wu *et al.*, 1987). A Superose 6 HR 10/30 column (Pharmacia Biotech) was used for all separations described in this work. Elution of LOS was monitored by refractive index, measured with a refractoMonitor* IV (LDC Analytical), and elution of nucleic acids by *A*₂₆₀ measured using a UV-MII monitor (Pharmacia). The LOS was recovered from pooled fractions by two rounds of ethanol precipitation as described above, once overnight and once for 6 h, and then freeze-dried and stored at −20 °C. This procedure has been shown to remove all sodium deoxycholate from LOS samples effectively (Wu *et al.*, 1987).

**Colorimetric and spectrophotometric estimations.** Total LOS was determined by the carbocyanine dye assay (Janda & Work, 1971), using 460 nm rather than 472 nm as the wavelength for absorbance measurements to reduce non-specific absorption (Johnson & Perry, 1976). The standard for this assay was LPS from *Salmonella minnesota* strain R5 (Rc mutant, Sigma). The RNA content of chromatography fractions and final LOS preparations (500 μg LOS in 0-1 M NaOH) was estimated by *A*₂₆₀ assuming 40 μg RNA ml⁻¹ to have a value of 1-0 (Sambrook *et al.*, 1989). Protein content was monitored by *A*₂₆₀.

**SDS-PAGE and immunoblotting.** Purified LOS was analysed in the discontinuous gel system, as described by Laemmli (1970), except that the stacking gel was 5% (w/v) polyacrylamide (Protogel, National Diagnostics), and the separating gel was 15% polyacrylamide, 4 M urea (Wu *et al.*, 1987). Samples of LOS (approx. 0-2 μg) were diluted to a volume of 10 μl with distilled water and mixed with an equal volume of solubilization buffer (Tsai & Frasch, 1982), heated to 100 °C for 5 min and then placed on ice before loading. Electrophoresis was at 15–20 mA, until the dye front migrated about 12 cm (approx. 5 h). The LOS bands were visualized by silver staining according to Tsai & Frasch (1982). For immunoblotting, approximately 2 μg of LOS was loaded onto duplicate SDS-PAGE gels. After electrophoresis, the gel was immediately silver-stained whilst the other was blotted onto a 0-2 μm nitrocellulose membrane using a dry blot system (Multiphor II NovaBlot, Pharmacia). The transfer was carried out using a continuous buffer system electrode solution recommended by the manufacturer [39 mM glycine, 48 mM Tris/HCl, 0.0375% (w/v) SDS; 20% (v/v) methanol] at 1 mA cm⁻¹ for 90 min. The blotted gel was silver-stained to check for completeness of transfer and the membrane was allowed to air-dry before blocking for 30 min in Tris-buffered saline (TBS; 20 mM Tris/HCl, pH 7-5; 500 mM NaCl) plus 1% (w/v) BSA. This was followed by overnight incubation with mAb at 4 °C, and 4-5 h at room temperature with peroxidase-conjugated goat anti-mouse IgG secondary antibody, both diluted with TBS plus BSA. The antibody incubation steps were followed by two 10 min washes in TBS plus 0-05% Tween 20. The substrate 4-chloro-1-naphthol was used to visualize LOS-antibody complexes [1:5 mixture of 3 mg 4-chloro-1-naphthol (ml methanol)⁻¹ and 0-018% H₂O₂ in 25 mM Tris/HCl, pH 7-4].

**ELISA.** The ELISA method used was essentially that of Verheul *et al.*, (1991). Purified LOS was resuspended at 10 μg ml⁻¹ in freshly-made bicarbonate buffer (15 mM Na₂CO₃, 35 mM NaHCO₃, pH 9-6), and 100 μl aliquots were used as the coating antigen for individual wells of a microtitre plate (incubated overnight at 37 °C). Plates were washed three times with 0-02% Tween 20 in PBS (Dulbecco ‘A’, pH 7-3), and incubated with 100 μl mAb per well, diluted in PBS containing 0-02% Tween 20 and 1% BSA, at 37 °C for 2 h. Binding was detected using a peroxidase-conjugated secondary antibody (goat anti-mouse IgG, whole molecule) and 3,3',5,5'-tetramethylbenzidine (TMB) as coloured substrate. The reaction was stopped by the addition of 100 μl 2 M H₂SO₄ and *A*₄₉₂ measured using a plate reader (Anthos Reader 2001, Anthos Labtec Instruments).

**Limulus amoebocyte lysate assay (LAL).** Endotoxic activity of the LOS was assessed using the microtitre LAL assay of Ray *et al.* (1991).
RESULTS

Chromatographic profile of LOS preparations

A chromatography profile of RNase A-treated, phenol-extracted meningococcal cell lysates separated on the Superose 6 column is shown in Fig. 1. The refractive index profile was reproducible and was identical for all samples examined, but the $A_{260}$ profile varied, depending on the RNase treatment applied to the sample before chromatography. Analysis of 0.5 ml fractions by SDSPAGE (data not shown), carbocyanine assay and ELISA (Fig. 2) demonstrated that the LOS was present in the fractions corresponding to an elution volume of 20-20.5 ml, coincident with the smaller of the refractive index peaks in Fig. 1. The large deflection in refractive index between elution volumes of 21 and 24 ml was probably caused by buffer components. The elution volume of the LOS corresponded to a $K_{av}$ of 0.7. This was comparable to the estimated $K_{av}$ of gonococcal LOS on a Sepharose CL-6B column (Rodahl & Maeland, 1984), reflecting the similarities between meningococcal and gonococcal LOS (Schneider et al., 1984; Mandrell et al., 1988), and contrasted with results obtained by gel filtration of meningococcal LOS using the dextran gel Sephadex G-75, which gave a $K_{av}$ of 0.2 (Wu et al., 1987).

The larger of the $A_{260}$ peaks, eluting between 16 and 24 ml in Fig. 1, had a $A_{260}/A_{280}$ ratio of greater than 2.0, indicating that this was most probably RNA. The smaller $A_{260}$ peak, around an elution volume of 10 ml, corresponded to a high $M_r$ band on a silver stained gel of the fractions (data not shown) and was probably residual polysaccharide. A minor peak in $A_{260}$, detectable by spectroscopic analysis, which eluted around a volume of 12 ml, probably corresponded to residual chromosomal DNA.

Alternative RNase treatments

Although RNase A had previously been used for reducing RNA contamination in chromatographically purified LOS (Wu et al., 1987; Gu & Tsai, 1991), Fig. 1 shows that, with the separation conditions used in the present work, most RNA eluted with the LOS resulting in substantial contamination of the final LOS preparation. To overcome this, untreated cell lysates or lysates subjected to digestion with RNase ONE were separated on the Superose 6 column under the same conditions. The $A_{260}$ and the activity in immunotype-specific ELISA of 0.5 ml fractions, corresponding to elution volumes of 15-24.5 ml, from each of these separations were determined (Fig. 3). The amount of RNA contamination of the LOS-containing fractions was less in untreated samples than in samples treated with RNase A. Treatment with RNase ONE substantially removed $A_{260}$-absorbing material from the LOS-containing fractions. Analysis of cell extracts with no digestion, RNase A digestion, and RNase ONE digestion by agarose gel electrophoresis showed that digestion with RNase ONE resulted in complete digestion of RNA whereas there was incomplete digestion of this material in the RNase A-treated sample (data not shown).

Yield and purity of LOS

The yield of crude material after freeze-drying was 15% (mg solids recovered per 100 mg estimated dry wt bacteria) without ribonuclease digestion and 10% after RNase ONE treatment. Approximately 80% of the LOS present in crude material applied to the column was recovered in the active fractions as measured by the carbocyanine assay, and the final yield after the chromatography step was 0.5-0.6% of initial estimated bacterial dry weight.

Samples of LOS-containing fractions from RNase ONE-treated lysates did not show any proteins on SDS-PAGE gels after silver staining when up to 50 μg of the
In cell lysates treated with RNase ONE before separation, less than 0.1 absorption units at 260 nm were detected in the chromatography fractions containing LOS, and after pooling and precipitation the final preparation obtained from such lysates was estimated to contain a maximum of 0.4% RNA. Samples of LOS prepared in a similar manner, but from crude material which had not undergone ribonuclease digestion, gave an absorption maximum equivalent to 2–3% RNA when the same three fractions were pooled.

**Biological activity of purified LOS**

The endotoxic activity of the purified LOS was examined by LAL assay. Specific activities \([\text{IU (µg LOS)}^{-1}]\) were as follows: cell lyse before treatment with RNase ONE, \(10 \times 10^3\); FPLC-purified, RNase ONE-treated LOS, \(17 \times 10^3\); FPLC-purified LOS without RNase treatment, \(14 \times 10^3\); and LPS from *Salmonella minnesota* strain R5, \(0.8 \times 10^3\). Immunological activities of crude and purified LOS were assessed by ELISA (Fig. 2) and immunoblotting (Fig. 4). Fig. 4 shows crude and purified LOS on SDS-PAGE gels, and after immunoblotting with the immunotype-specific mAb 9-2-L379. Both samples appeared identical on the gel and both reacted equally well with the mAb.

**DISCUSSION**

Hot phenol extraction of whole cells (Westphal & Jann, 1965) or cell lysates (Johnson & Perry, 1976) can be used to prepare samples of the LOS of a number of *Neisseria* species. However, whilst these preparations are adequate for electrophoretic analyses, they contain RNA as their main component and require further purification before use in molecular and immunological studies. Gel filtration on a Superose 6 column using the FPLC system is a rapid, reproducible method for achieving this.

For optimal LOS purification, it was necessary to replace RNase A, which is normally included in protocols for the purification of LOS by conventional gel filtration (Wu et al., 1987; Gu & Tsai, 1991), with RNase ONE. The advantage of RNase ONE over RNase A, which degrades RNA to oligoribonucleotides (Kalintsky et al., 1959), is that its digestion products are mononucleotides (Meador et al., 1990). Agarose gel electrophoresis of cell extracts treated with RNase A and RNase ONE confirmed that the RNA A-treated samples contained RNA of a size range not present in the RNase ONE treated samples. On separation on the Superose 6 column, RNase ONE-digested samples contained less material with an absorbance of 260 nm than the RNase A-treated samples. In addition, the RNase ONE digestion products did not co-elute with the LOS.

The recovery of solids from RNase ONE-treated cell lysates (10%) and untreated cell lysates (15%) was similar to previously reported values (Johnson & Perry, 1976; Westphal & Jann, 1965; Romanowska & Mulczyk, 1968), but the recovery of the LOS from the gel filtration step on the Superose 6 column (80%) was slightly higher than that obtained with conventional gel filtration (Rodahl &

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**Fig. 3.** Separation of phenol-extracted cell lysates treated (a) or untreated (b) with RNase ONE. The \(A_{260}\) of samples diluted 1:20 with elution buffer (□) and the LOS activity in ELSA with the immunotype-specific mAb 9-2-L379 of fraction samples diluted 1:10, with the background subtracted (●) are shown. The results given are representative values from a single experiment.

**Fig. 4.** Comparison of LOS before and after separation on a Superose 6 column. (a) Silver-stained SDS-PAGE gel. Lanes: 1, crude LOS (2 µg); 2, purified LOS (2 µg). (b) Immunoblot of a gel identical to that shown in (a) with immunotype-specific mAb 9-2-L379.

LOS sample was loaded per well. The silver stain method used was capable of detecting nanograms of proteins (Morrisssey, 1981), and on the basis of this figure, the maximum level of contamination of the final LOS preparation by any particular protein was estimated as less than 0.01% by mass. Samples of 500 µg purified LOS ml\(^{-1}\) had an \(A_{280}\) of 0.001, giving an estimated maximum protein contamination of 0.2%, assuming 1 mg protein ml\(^{-1}\) to have an \(A_{280}\) of 1.0.
Maeland, 1984). The final yield of LOS (0.5–0.6%) was similar to, and levels of RNA contamination less than, those obtained by others (Wu et al., 1987; Gu & Tsai, 1991). Protein contamination of the LOS (less than 0.2%) was lower than with previously reported methods (Wu et al., 1987; Gu & Tsai, 1991). However, it is possible that in the former studies the levels of protein contamination were over-estimated, as at least one of the protein assays used gives positive reactions with reagents such as EDTA and sodium deoxycholate (Markwell et al., 1981) and the hexosamines and lipids present in the LOS can generate inaccuracies in protein determinations (Herd, 1971; Eichberg & Mokrasch, 1969). For this reason we have used spectrophotometric determinations and silver staining of SDS-PAGE gels to estimate the levels of protein contamination present.

Biological activity of the LOS was not lost during purification: the purified LOS was highly endotoxic compared to the reference sample in the LAL test and had the highest specific activity of all of the samples examined. The immunological activities of crude and purified LOS as assessed by ELISA and immunoblotting were indistinguishable. Samples of LOS prepared from two other N. meningitidis strains by this method were highly toxic to human endothelial cells in an in vitro toxicity model (Dunn et al., 1995).

Previously, LOS was purified by ultracentrifugation (Westphal & Jann, 1965; Romanowska & Mulczyk, 1968; Perry et al., 1975; Jennings et al., 1980; Brade & Galanos, 1982; Darveau & Hancock, 1983; Pepper, 1984; Jennings et al., 1984; Inzana et al., 1985; Kulshin et al., 1992) and conventional gel filtration (Romanowska, 1970; Wu et al., 1987; Gu & Tsai, 1991, 1993; Gu et al., 1992; Pavliak et al., 1993). However, ultracentrifugation is time-consuming and results in poor yields while conventional gel filtration requires long chromatography runs and the processing of relatively large numbers and volumes of collected fractions. The separation of RNase ONE-treated cell extracts with a FPLC system and a Superose 6 column avoids these problems and is an efficient alternative procedure for the purification of pure, active LOS.

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