Chemical and Biological Properties of Lipopolysaccharide, Lipid A and Degraded Polysaccharide from *Wolinella recta* ATCC 33238

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Lipopolysaccharide (LPS) was isolated and purified from *Wolinella recta* ATCC 33238 by the phenol–water procedure and RNAase treatment. The sugar components of the LPS were rhamnose, mannose, glucose, heptose, 2-keto-3-deoxyoctonate (KDO) (3-deoxy-D-manno-octulosonate) and glucosamine. The degraded polysaccharide prepared from LPS by mild acid hydrolysis was fractionated by Sephadex G-50 gel chromatography into three fractions: (1) a high-molecular-mass fraction, eluting just behind the void volume, consisting of a long chain of rhamnose (22 mols per 3 mols of heptose residue) with attached core oligosaccharide; (2) a core oligosaccharide containing heptose, glucose and KDO, substituted with a short side chain of rhamnose; (3) a low-molecular-mass fraction containing KDO and phosphate. The main fatty acids of the lipid A were C_{12:0}, C_{14:0}, 3-OH-C_{14:0} and 3-OH-C_{16:0}. The biological activities of the LPS were similar to those of *Salmonella typhimurium* LPS in activation of the clotting enzyme of *Limulus* amoebocytes, the Schwartzman reaction and mitogenicity for murine lymphocytes, although all the biological activities of lipid A were lower than those of intact LPS.

INTRODUCTION

Previous evidence has shown that the characteristic microbial complexes from different forms of destructive periodontal diseases play a specific aetiological role. For instance, *Bacteroides gingivalis* is associated with advanced destructive periodontitis in certain individuals (Slots, 1982) and *Bacteroides intermedius* with acute necrotizing ulcerative gingivitis (Chung *et al.*, 1983). In lesions of juvenile periodontitis, *Actinobacillus actinomycetemcomitans* is particularly likely to be detected (Zambon *et al.*, 1983). On the other hand, several investigators showed that the proportion of motile rods is much higher in active periodontal disease sites than in inactive sites (Listgarten, 1982; Savitt & Socransky, 1984).

Tanner *et al.* (1984) recently isolated an asaccharolytic, Gram-negative, motile rod, which was named *Wolinella recta*, from periodontal pockets of patients with advanced destructive periodontal disease. In some lesions, this bacterium was isolated as the numerically dominant species (Moore *et al.*, 1982, 1984; Dzink *et al.*, 1985); however, there is no direct evidence of the periodontopathic potential of this bacterium. Grenier & Mayrand (1986) also reported nutritional relationships between *W. recta* and *B. gingivalis* or *Bacteroides melaninogenicus*; for instance, *W. recta* produces a protohaem that stimulates the growth of *B. gingivalis* or *B. melaninogenicus*, and *B. melaninogenicus* produces formate, which stimulates the growth of *W. recta*.

Abbreviations: DPS, degraded polysaccharide; KDO, 2-keto-3-deoxyoctonate (3-deoxy-D-manno-octulosonate).

It has been speculated that numerous surface components of periodontopathic bacteria act as virulence factors that probably cause host tissue destruction in periodontal disease. In the present study, the chemical composition and biological activity of *W. recta* lipopolysaccharide (LPS) together with its degraded polysaccharide and lipid A were investigated.

A preliminary account of this study was published by Kumada *et al.* (1987).

METHODS

Organism and cultivation. *W. recta* ATCC 33238 was grown to early stationary phase in Schaedler broth (BBL Microbiology Systems) supplemented with formate (0.2%, w/v) and fumarate (0.3%, w/v) at 37 °C in an anaerobic chamber containing 70% N₂, 15% H₂ and 15% CO₂ (by vol.). The bacterial cells were inactivated by heating at 121 °C for 20 min, and then washed three times with distilled water and dried with acetone.

Preparation of LPS. LPS was extracted from the acetone-dried cells by the phenol/water technique of Westphal *et al.* (1952). It was purified by repeated ultracentrifugation (105000 g for 10 h) after each washing before and after RNAase treatment (Westphal & Jann, 1966) and was then lyophilized. LPSs of *Salmonella typhimurium* LT2 and *Salmonella minnesota* R595 were obtained by Westphal's phenol/water method and Galanos's phenol/chloroform/light petroleum method (Galanos *et al.*, 1969), respectively.

Degradation of LPS. LPS was hydrolysed with 5% (v/v) acetic acid (10 mg ml⁻¹) at 100 °C for 7.5 h; the resulting insoluble material was removed by centrifugation (15000 g, 10 min), washed three times with distilled water and then lyophilized to obtain lipid A. The supernatant and the first washings were combined and rotary-evaporated under reduced pressure to dryness yielding degraded polysaccharide (DPS). Yields of DPS and lipid A from LPS were 76.1% (w/w) and 16.9% (w/w), respectively.

Analytical methods. Total carbohydrate was measured by the phenol/sulphuric acid method (Dubois *et al.*, 1956), and total phosphorus and amino sugar were determined as described by Hisatune *et al.* (1967). Heptose was determined either by the method of Osborn (1963) or by GLC. Protein was assayed by the method of Bradford (1976). 2-Keto-3-deoxyoctonate (KDO) (3-deoxy-D-manno-octulosonate) was estimated by the conventional Weissbach periodate/thiobarbituric acid reaction under conventional hydrolysis conditions (0.01 M-H₂SO₄ at 100 °C for 20 min) (Weissbach & Hurwitz, 1959). KDO (ammonium salt) used as a standard was purchased from Sigma. Total lipid was measured by weighing the ether-soluble fraction after hydrolysis of LPS in 4 M-HCl at 100 °C for 4 h.

Neutral sugars were determined after hydrolysis in 2 M-trifluoroacetic acid at 120 °C for 1 h by GLC as alditol acetates according to the method of Laine *et al.* (1974) (column: 3% ECNSS-M coated on Chromosorb W, 3 mm × 2 m; Gasukuro Kogyo Co., Japan) using xylose as an internal standard. For amino sugar determination, samples were hydrolysed in 4 M-HCl at 100 °C for 8 h, and the hydrolysates were fractionated on a Dowex 50 (H⁺) column by the method of Wheat (1966). The amino sugar fraction thus separated was *N*-acetylated by the method of Laine *et al.* (1974). Amino sugars were then determined as *N*-acetylalditol acetates by GLC (column: TABSORB, 3 mm × 2 mm; Regis Chemical Co., USA) using mannose as an internal standard. Fatty acids released by heating in 4 M-HCl at 100 °C for 4 h and then in 1 M-NaOH at 100 °C for 2 h (Haeffner *et al.*, 1977) were esterified with diazomethane (Rietschel *et al.*, 1972) and then analysed by GLC (column: 25% DEGS, 3 mm × 2 m; Gasukuro Kogyo Co.). Methylnonadecanoate was used as an internal standard. Neutral and amino sugars and fatty acids were identified by their retention times in GLC and their mass spectra in GLC/MS as compared with those of authentic standards. GLC/MS spectrometry was done on a JEOL DX-300 with the same column as described above. All analytical data are expressed as the mean values of three experiments.

Gel filtration of DPS (110 mg) was done with a Sephadex G-50 column (1.6 × 100 cm) with pyridine/acetic acid/water (10:4:1000, by vol.) used as solvent at a flow rate of 13 ml h⁻¹; fractions (1 ml) of the eluate were collected. The eluate was monitored with a refractive index detector (RID-6A; Shimadzu).

SDS-PAGE. This was done according to the method of Laemmli (1970) using slab gels (14 × 12 cm). The separating gel contained 14% (w/v) acrylamide and the stacking gel contained 4% (w/v) acrylamide. LPS samples in water were mixed with an equal volume of 0.1 M-Tris/HCl buffer (pH 6.8) containing 2% (w/v) SDS, 20% (w/v) sucrose, 1% (w/v) 2-mercaptoethanol and 0.001% (w/v) bromophenol blue. The mixtures were incubated for 5 min at 100 °C, and 10 µl samples were applied to each well of the gel and subjected to electrophoresis at constant current (30 mA per gel) until the dye had migrated to the bottom of the gel. LPSs on the gel were detected by the silver stain method of Tsai & Frasch (1982).

Limulus test. For *Limulus* amoebocyte lysate clotting activity, 200 µl of Toxicolour system reagent (LS-20 set; Seikagaku Kogyo Co., Japan) (Iwanaga *et al.*, 1978) and 20 µl of diluted samples (LPS, DPS and lipid A) were incubated for 30 min at 37 °C. Lipid A was dissolved in 0.5% (v/v) triethylamine. The reaction was stopped by placing the tube in an ice-bath and adding 0.4 ml 0.8 M-acetic acid, and the *A*₄₀₅ was then measured. The minimum dose of LPS for a positive reaction was determined as the amount giving an *A*₄₀₅ > 0.1. *S. typhimurium* LPS (Sigma) was used as positive control.

Schwartzman assay. The dermal Schwartzman assay was done by injecting three male Japanese White rabbits (1.5 to 2.0 kg) intradermally in the shaved abdomen with 1, 10 and 100 µg doses of samples in 0.1 ml of Dulbecco's phosphate-buffered saline (Nissui Pharmaceutical Co., Japan), followed 24 h later by a provoking intravenous injection of 100 µg *S. typhimurium* LPS in 0.1 ml of Dulbecco's phosphate-buffered saline. The injection sites were examined for haemorrhagic necrosis 5 h after injection of the provoking dose.

Mitogenic activity. Spleen cells from C3H/HeJ and C3H/HeN mice were suspended in RPMI 1640 medium (Gibco) supplemented with penicillin (100 U ml⁻¹), streptomycin (100 µg ml⁻¹) and 20% (v/v) foetal calf serum (Gibco). Lymphoid cells were cultured in a 96-well microculture plate containing various amounts of samples or concanavalin A (Sigma), at 37 °C in a humidified 5% (v/v) CO₂ atmosphere. Cultures were pulsed with 0.5 µCi (18.5 kBq) [³H]thymidine ([³H]TdR) [18.2 Ci mmol⁻¹ (673 TBq mmol⁻¹); New England Nuclear] during the final 24 h of a 72 h incubation period. Cells were then harvested, and the incorporation of [³H]thymidine by the cultured cells was measured with a liquid scintillation counter. The minimum mitogenic response was set as that in which the stimulation index was higher than the index of the maximum response of C3H/HeJ mouse spleen cells when subjected to the range of concentrations of LPS tested.

RESULTS AND DISCUSSION

Extraction yield and overall chemical composition

In the preliminary experiments, the yield of crude LPS was small when extracts were ultracentrifuged for 3 h. The period of centrifugation was therefore extended to 10 h. The crude LPS preparations obtained by ultracentrifugation for 3 and 10 h showed the same chemical composition overall and the same content of neutral and amino sugars determined by GLC. The yield of the purified LPS from the acetone-dried cells was 0.9% (w/w). When the purified LPS, dissolved in distilled water, was scanned from 190 to 310 nm, no absorption peak at either 260 nm or 280 nm was observed, indicating that, as far as UV absorption showed, no nucleic acid or protein was present in the LPS as contaminant.

The overall chemical composition of *W. recta* LPS is shown in comparison with that of *S. typhimurium* LT2 in Table 1. The composition of the two LPSs was virtually the same although the total lipid (16.5%) of the former was lower than that of the control LPS (28.9%). Thus, the overall chemical composition of the *W. recta* LPS was not very different from that of a typical Gram-negative bacterial LPS.

Sugar composition

In a preliminary experiment, when LPS was treated with 1% acetic acid for 12 h at 100 °C, lipid A and DPS were not cleaved. To hydrolyse the LPS, it was necessary to use 5% acetic acid at 100 °C for 7.5 h. The results of the sugar analysis of the LPS are in Table 2. Rhamnose, mannose, glucose, L-glycero-D-mannoheptose, D-glycero-D-mannoheptose, KDO and glucosamine were found. Glucose, L-glycero-D-mannoheptose and KDO are usual sugar constituents of the core region of typical Gram-negative bacterial LPS, whereas glucosamine is a basic structural unit of the lipid A backbone of LPS (Rietschel *et al.*, 1982, 1984). It was particularly noted that as much as 29.8% rhamnose was found in the LPS of *W. recta*. Furthermore, in addition to L-glycero-D-mannoheptose (5.2%), D-glycero-D-mannoheptose, which has rarely been reported as a component sugar of Gram-negative bacterial LPS (Wilkinson, 1977), was found in considerable amounts (3.0%), the molar ratios of the two heptoses being 2:1.2.

Gel filtration of DPS

The distribution of the component sugars between the core and O-specific side-chain regions can often be determined by fractionating the DPS, isolated from LPS by mild acid hydrolysis, by gel filtration (Wilkinson, 1977). As shown in Fig. 1, the elution profile for *W. recta* DPS was almost the same as that of the DPS of the typical Gram-negative bacterial LPS (Wilkinson, 1977), with three distinct peaks being eluted. If the results obtained by this gel filtration can be interpreted in the same way as those of other, similar experiments (reviewed by Wilkinson,

Table 1. Chemical composition (% w/w) of LPS from *W. recta* and *S. typhimurium*

LPS	Protein	Total carbohydrate	Amino sugar	Total phosphorus	Total lipid
<i>W. recta</i> ATCC 33238	3.6	53.2	6.8	0.8	16.5
<i>S. typhimurium</i> LT2	3.0	53.0	3.7	2.3	28.9

Table 2. Chemical composition (% w/w) of LPS, DPS and lipid A from *W. recta*

The data in parentheses represent the molar ratios calculated on the assumption that each fraction contains 3 mols of heptose.

LPS fraction	Rhamnose	Mannose	Glucose	Heptose			KDO	Glucosamine	Total phosphorus	Total lipid	Yield of fraction from LPS
				L-Glycero- and D-glycero-D-mannoheptose							
LPS (undegraded)	29.8 (14.0)	0.6 (0.3)	0.9 (0.4)	8.2 (3.0)	3.7 (1.1)	2.0 (0.7)	16.5	0.8	16.5	—	
DPS											
Fraction I	47.3 (21.6)	1.8 (0.8)	1.9 (0.8)	8.4 (3.0)	0.5 (0.1)	tr.	ND	tr.	tr.	76.1 37.7	
Fraction II	19.1 (5.0)	0.6 (0.1)	3.4 (0.8)	15.1 (3.0)	0.8 (0.1)	tr.	ND	0.1	ND	11.8	
Fraction III	2.7	tr.	0.2	2.0	13.3	0.4	ND	4.0	ND	4.9	
Lipid A	1.2	tr.	tr.	ND	ND	7.7	1.5	1.5	53.4	16.9	

tr., Trace amount (<0.1%).
ND, Not determined.

tr., Trace amount (<0.1%).
ND, Not determined.

Fig. 1. Sephadex G-50 gel chromatography of the DPS fraction isolated from *W. recta* LPS. The elution profile is presented as the refractive index detector recorder response. The fractions shown by each horizontal line were pooled.

1977), the first peak (fraction I) corresponds to the polymeric *O*-side-chain with attached core, the second peak (fraction II) to the core oligosaccharide and the third peak (fraction III) to the monosaccharide fraction that contains KDO.

The results of sugar analyses of the three fractions obtained from DPS are in Table 2. Fraction II contained rhamnose, glucose and heptose in a molar ratio of 5:1:3 as the major sugar components and trace amounts of mannose and KDO, while fraction I contained, as predominant sugar components, rhamnose, mannose, glucose and heptose in a molar ratio of 22:1:1:3 and trace amounts of KDO. These results indicate that the *O*-specific chain of the LPS of this species consists of as much as 22 mols of rhamnose per 3 mols of heptose, while the core region consists of 1 mol of glucose, 3 mols of heptose and presumably 1 mol or more of KDO. Furthermore, GLC analysis revealed that the heptose component of fraction I and II consisted of L-glycero- and D-glycero-D-mannoheptose in molar ratios of 2:1.2 and 2:1.1, respectively. Thus, 2 mols of L-glycero-D-mannoheptose and 1 mol of D-glycero-D-mannoheptose are present as the heptose constituent in the core region of *W. recta* LPS.

With regard to the mannose component, it is hard to explain why only trace amounts of mannose were found in fraction II corresponding to the core whereas 1 mol of mannose was found in fraction I corresponding to the *O*-specific side-chain with attached core. In fraction II, 5 mols of rhamnose per 3 mols of heptose were found, suggesting that the core oligosaccharide substituted with a short chain of rhamnose was eluted in this fraction, but not in fraction I, probably because of its small molecular size. Fraction III contained free KDO (13.3%), released from LPS during treatment with 5% acetic acid for the preparation of DPS.

SDS-PAGE analysis

Electrophoretic banding patterns of LPS in SDS-PAGE have been useful in studying the heterogeneity and structure of LPS molecules of several genera of Gram-negative bacteria (Hitchcock *et al.*, 1986). As shown in Fig. 2, *S. typhimurium* LT2 LPS (lane 2) showed the characteristic pattern that originates from the number of repeating units composed of *O*-specific side-chains in the LPS molecules, at least 30 bands being observed with the smooth-type LPS. This result was consistent with that of Goldman & Leive (1980). *S. minnesota* R595 (Re mutant) LPS consisting of lipid A and three molecules of KDO showed only a single rapidly migrating band (Fig. 2, lane 3). The electrophoretic behaviour of *W. recta* LPS (Fig. 2, lane 1) differed from that of LT2 LPS in that only a fast-migrating band and a few bands near the middle of the gel were observed. The fast-migrating band was believed to correspond to the LPS molecule lacking an *O*-specific side-chain and the few bands near the middle of the gel to correspond to smooth-

Fig. 2. SDS-PAGE patterns of LPSs from *W. recta* ATCC 33238 (1 μ g; lane 1), *S. typhimurium* LT2 (8 μ g; lane 2) and *S. minnesota* R595 (Re mutant) (4 μ g; lane 3).

type LPS possessing an *O*-specific side-chain. Furthermore, from a comparison of the patterns obtained by SDS-PAGE and by gel filtration for *W. recta* LPS, it was considered that the fast-migrating band in SDS-PAGE represents fraction II (core fraction) in gel filtration, and that the middle bands represent fraction I (*O*-specific side-chain fraction with attached core). The molecular size of the core region of *W. recta* LPS, containing 5 mols of rhamnose per 3 mols of heptose, was slightly bigger than that of *S. typhimurium* LT2 LPS as the front band in SDS-PAGE for *W. recta* LPS had a lower mobility than that of *S. typhimurium* LT2 LPS.

As described above, the *O*-specific side-chain of *W. recta* LPS contained a polymer of rhamnose. It is well-known that a number of ladder bands are detected by SDS-PAGE for smooth-type LPS of *S. typhimurium* because individual LPS molecules with *O*-specific side-chains differing in size by multiple increments of oligosaccharide repeating units (Hitchcock, 1983). However, this characteristic heterogeneity was not seen with *W. recta* LPS. These results suggest that the chain-lengths of the *O*-specific side-chains of *W. recta* LPS are considerably more homogeneous than those of *S. typhimurium* LT2 LPS.

Fatty acid composition of lipid A

Lipid A contained 53% total lipid (Table 2). The non-hydroxylated fatty acids found were as follows: $C_{12:0}$ (5.5%) and $C_{14:0}$ (16.2%) as the major components, and small amounts of $C_{14:1}$ (2.4%), $C_{16:0}$ (1.5%) and $C_{16:1}$ (1.8%) as additional minor components. In addition, large

Table 3. Minimum doses of LPS, lipid A and DPS inducing *Limulus* amoebocyte lysate clotting activity and the Schwartzman reaction

Stimulant	Minimum inducing dose	
	<i>Limulus</i> (pg ml ⁻¹)	Schwartzman (µg per site)
<i>W. recta</i> ATCC 33238		
LPS	10	10
Lipid A	100	100
DPS	100000	—*
<i>S. typhimurium</i> LPS	100	10

* No haemorrhagic necrosis induced by a dose of 100 µg per site.

Table 4. Mitogenic responses of C3H/HeN mouse spleen cells to LPS, lipid A and DPS

Data are expressed as the mean of the stimulation index $\pm$ SE of triplicate cultures. Concanavalin A (5 µg ml⁻¹) was mitogenic for C3H/HeN spleen cells (39.0 $\pm$ 2.7).

Stimulant	Mitogenic response			
	Dose (ng ml ⁻¹):			
	1	10	100	1000
<i>W. recta</i> ATCC 33238				
LPS	0.9 $\pm$ 0.1	1.3 $\pm$ 0.1	3.6 $\pm$ 0.1	10.8 $\pm$ 0.6
Lipid A	0.9 $\pm$ 0.1	0.9 $\pm$ 0.1	1.4 $\pm$ 0.1	5.4 $\pm$ 0.4
DPS	1.1 $\pm$ 0.1	1.0 $\pm$ 0.0	1.0 $\pm$ 0.1	1.1 $\pm$ 0.2
<i>S. typhimurium</i> LPS	1.9 $\pm$ 0.2	7.3 $\pm$ 0.6	7.7 $\pm$ 2.0	13.1 $\pm$ 0.2

amounts of two hydroxylated fatty acids, 3-OH-C_{14:0} (29.5%) and 3-OH-C_{16:0} (43.1%), were found. *W. recta* lipid A therefore resembles the lipid A of the Gram-negative obligately anaerobic filamentous bacterium *Fusobacterium nucleatum*, which also contains the above hydroxylated fatty acids as major components. This is of interest as lipid A is one of the potential pathogenic factors in periodontal disease and *F. nucleatum* is prominent in the bacterial flora of subgingival plaques. However, *W. recta* lipid A also contained glucosamine and total phosphorus in an approximate molar ratio of 1:1 (Table 2), indicating that it probably possesses the same diglucosaminide backbone phosphorylated at the 1 and 4' positions as that found commonly in the lipid A of the LPS of Enterobacteriaceae.

Biological activities

The endotoxic activities of LPS, DPS and lipid A from *W. recta* were examined using the *Limulus* amoebocyte and dermal Schwartzman tests. The results are expressed as the coincident values in triplicate experiments (Table 3). *S. typhimurium* LPS was used as a control in both bioassays.

In the *Limulus* amoebocyte test LPSs from *W. recta* and *S. typhimurium* strongly activated the clotting enzyme; the minimum doses for these LPS-induced positive reactions were 10 and 100 pg ml⁻¹, respectively. However, *W. recta* lipid A had a slightly weaker activity (100 pg ml⁻¹) than the LPS, and the *W. recta* DPS did not significantly activate the enzyme at the concentration (100 ng ml⁻¹) used.

In the dermal Schwartzman reaction, the minimum doses of LPSs from both *W. recta* and *S. typhimurium* that caused haemorrhagic necrosis were 10 µg per site; the minimum dose (100 µg per site) of lipid A was lower than that of *W. recta* LPS. Moreover, the DPS of *W. recta* did not induce the Schwartzman phenomenon.

The mitogenic activities of *W. recta* LPS, DPS and lipid A, and *S. typhimurium* LPS as a positive control, were determined in spleen cells of C3H/HeN and C3H/HeJ mice. The results

are expressed as the arithmetic mean of the stimulation index (c.p.m. for tested culture/c.p.m. for culture with medium alone = 329 ± 24 c.p.m.). All fractions except *W. recta* DPS were strongly mitogenic for C3H/HeN murine spleen cells at concentrations between 10 ng ml^{-1} and $1 \text{ } \mu\text{g ml}^{-1}$ (Table 4), whereas none were mitogenic for spleen cells of C3H/HeJ mice at any dose tested. Similarly to the other two endotoxic activities, the minimum dose of lipid A ($1 \text{ } \mu\text{g ml}^{-1}$) causing a positive reaction was lower than that of *W. recta* LPS (100 ng ml^{-1}); DPS was not mitogenic at the doses used. *W. recta* LPS was strongly mitogenic for BALB/c and BALB/c (nu/nu) murine spleen cells (data not shown). The above results indicate that *W. recta* LPS is a B-cell mitogen in murine spleen cells.

These immunological characters of *W. recta* LPS may play aetiological roles in the progression of periodontal disease.

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