Chemical Characteristics of Endotoxin from *Bacteroides fragilis* NCTC 9343

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SUMMARY

Phenol-water extracted lipopolysaccharides (LPS) from *Bacteroides fragilis* NCTC 9343 did not contain heptose or 2-keto-3-deoxy-octonate. The sugar components identified were glucosamine, galactosamine, glucose, galactose, fucose, rhamnose and traces of mannose. An unidentified amino compound was found. The preparations were free of nucleic acids and protein constituted only a minor fraction of the LPS. The LPS preparations had endotoxic effect when tested in rabbits, but the endotoxic potency was low.

INTRODUCTION

Endotoxic lipopolysaccharide (LPS) from *Bacteroides melaninogenicus* lacks heptose and 2-keto-3-deoxy-octonate (KDO) (Hofstad, 1968). With a few exceptions (Hickman & Ashwell, 1966; Volk, 1966; Kasai & Nowotny, 1967; Adams, Tornabene & Yaguchi, 1969) these sugars are present as essential constituents in LPS from aerobic Gram-negative bacteria (Osborn, 1963; Lüderitz, Staub & Westphal, 1966). The unexpected finding that both sugars were absent in *B. melaninogenicus* LPS has prompted a survey of the chemistry of LPS from related non-sporing anaerobic Gram-negative bacteria. The present report deals with the chemical composition of LPS from *B. fragilis* NCTC 9343.

METHODS

Bacterial strains. The *Bacteroides fragilis* strain NCTC 9343 was furnished by Dr Ella Barnes, Food Research Institute, Norwich, England. Cultivation was performed as described earlier (Hofstad, 1968) in an enriched fluid medium based on beef extract.

Extraction of LPS. Extraction of LPS with phenol-water (Westphal, Lüderitz & Bister, 1952) and further purification by ultracentrifugation were carried out as before (Hofstad, 1968). Extractions were made at room temperature from acetone-dried whole cells. After ultracentrifugation (100,000 *g* for 1 hr) the pellet was suspended in 0.1 M phosphate buffer pH 7.0 containing ribonuclease (5 × cryst., ex-bovine pancreas, Sigma Chemical Company, St Louis, Missouri, U.S.A.) and deoxy-ribonuclease (25 % activity of crystalline material deoxyribonuclease, ex-bovine pancreas, L. Light & Co. Ltd., Colnbrook, England) to give an enzyme:substrate ratio of about 1:50, and incubated at 37° for 1 hr. The ultracentrifugation was repeated, and the washed LPS finally taken up in distilled water and lyophilized.

Paper chromatography. Acid hydrolysis was performed in sealed tubes at 100° with 4 N-HCl for 4 hr, or with N-H₂SO₄ for 10 min., 4 or 16 hr, as required. Acid was removed from HCl hydrolysates by evaporation *in vacuo* in the presence of NaOH pellets. The H₂SO₄ hydrolysates were neutralized by passage through a column of Dowex-1 in the formate form. Circular paper chromatography was carried out with *n*-butanol + pyridine + water (6 + 4 + 3) or *n*-propanol + 1 % aqueous ammonium hydroxide (7 + 3). Sugars were stained with silver nitrate or aniline hydrogen phthalate. Amino sugars were also detected with the Elson-Morgan reagent (Partridge, 1948) or with ninhydrin.

Chemical analyses. Nitrogen was determined by the Micro-Kjeldahl method as described by Kabat & Mayer (1961). Samples were digested for 4 hr. Hexosamines were estimated with the Randle & Morgan method (1955), with glucosamine.HCl as standard. Samples were hydrolysed at 100° with 3 N-HCl for 4 hr. Amino sugars were also separated on columns of Dowex-50 w × 8 (Gardell, 1953). Protein was estimated by the Folin-Ciocalteu phenol method (Lowry, Rosebrough, Farr & Randall, 1951) with bovine serum albumin as standard. Fatty acid esters were determined as tripalmitin by the method of Snyder & Stephens (1959). Neutral sugars were measured by the Winzler orcinol method (Winzler, 1955) with glucose + galactose (1 + 1) as standard. Methyl pentose was estimated by the sulphuric acid + cysteine method as described by Dische (1955). The sulphuric acid + cysteine reaction was also used as a test for heptoses (Dische, 1953). The presence of KDO was investigated by the malonealdehyde + thiobarbituric acid method as modified by Weissbach & Hurwitz (1959).

Shwartzman reaction. Albino rabbits of either sex, 6 months old, and weighing 2.6–3.7 kg. were used. The animals were fed commercial pellets and water *ad libitum*. Serial dilutions of LPS were made in sterile saline. Doses of 0.2 ml. of each dilution were injected intradermally on alternative sites into the shaved abdomen. Provocative injections of LPS were given 24 hr later, and the animals were examined for haemorrhagic lesions on the following day.

RESULTS

Quantitative chemical analyses were carried out on three different batches obtained after extraction of acetone-dried cells with phenol-water for 15 or 30 min. (Table 1). Extraction for 15 min. gave a product which contained only small amounts of protein. The u.v. absorption curve of this LPS showed no peak or shoulder in the region of 260 nm. indicating that the LPS was free of or virtually free of nucleic acids. The yield was rather low, varying between 1 and 2 %. By extending the extraction to 30 or 45 min. the yield could be more than doubled. However, LPS obtained by such prolonged extraction was contaminated with protein, and possibly also with intracellular glycogen as judged by a relatively strong glucose spot on chromatograms made with acid hydrolysates.

Chromatographic analysis of sulphuric acid hydrolysates (N-H₂SO₄, 4 hr) of LPS obtained after extraction for 15 or 30 min. revealed bands corresponding to glucose, galactose, fucose, rhamnose and traces of mannose. Glucosamine and galactosamine were identified in hydrochloric acid hydrolysates. In addition, an unknown amino compound with an $R_{\text{glucosamine}}$ value of 1.64 in *n*-butanol + pyridine + water was detected on paper chromatograms developed with the Elson-Morgan reagent. The

Fig. 1. Elution pattern of amino sugars and the unidentified amino compound from *Bacteroides fragilis* (Batch 3, 40 mg.) on a Dowex-50 column. Each fraction contained 2 ml. Amino sugar concentrations are quoted as though they were glucosamine. A, glucosamine; B, galactosamine; C, unidentified amino sugar.

Table 1. Chemical composition of lipopolysaccharide isolated from *Bacteroides fragilis* NCTC 9343 by extraction of acetone-dried cells with phenol-water for 15 min. (Batch 1 and 2) or 30 min. (Batch 3)

	Neutral sugar (%)	Methyl- pentose (%)	Hexosamine (%)	Fatty acid ester (%)	Nitrogen (%)	Protein (%)
Batch 1	27.1	8.9	14.8	24.7	2.2	3.4
Batch 2	29.3	9.3	14.8	21.6	2.3	2.6
Batch 3	32.9	3.7	7.3	14.6	6.0	40.3

compound reduced silver nitrate, but gave no colour reaction with aniline hydrogen phthalate. On Dowex-50 the compound had a chromatographic mobility of 1.37 relative to glucosamine when eluted with 0.3 N-HCl (Fig. 1).

KDO could not be demonstrated when unhydrolysed samples, or samples of LPS hydrolysed for varying periods of time with 0.02 N-sulphuric acid, were examined by the thiobarbituric acid method.

Unhydrolysed samples of LPS were examined in the sulphuric acid-cysteine reaction of Dische (1953). The chromogenes gave an absorption maximum at 400 nm., and smaller peaks at 540 and 600 nm. No peak was observed at 510 nm., thus excluding the presence of heptose, nor was heptose demonstrated in the 16 hr sulphuric acid hydrolysates by paper chromatography. Acid labile deoxysugars were not detected in samples of LPS hydrolysed with N-sulphuric acid for 10 min.

The endotoxic potency of LPS was estimated by the local Shwartzman reaction in 12 rabbits. The animals were each prepared with different doses of LPS obtained by

extraction for 15 min. (6 rabbits) or for 30 min. (6 rabbits). Provocation was performed by intravenous administration of 400 µg. LPS. The results have been listed in Table 2.

Table 2. *Local Shwartzman reaction elicited by lipopolysaccharide isolated from Bacteroides fragilis* NCTC 9343 by extraction of acetone-dried cells with phenol-water for 15 min. (Batch 1) or 30 min. (Batch 3)

	Preparative injection (µg.)						Saline
	400	200	100	50	25	12.5	
Batch 1	6/6*	6/6	3/6	1/6	0/6	0/6	0/6
Batch 3	4/6	3/6	3/6	3/6	1/6	0/6	0/6

* Positive Shwartzman reaction/total, 18 hr after provocative injection.

DISCUSSION

The outstanding feature of the present study is the lack of heptose and KDO in LPS prepared from *Bacteroides fragilis* NCTC 9343. These constituents are also lacking in LPS isolated from oral strains of *B. melaninogenicus* (Hofstad, 1968). This suggests that endotoxic LPS of *Bacteroides* species has a core structure differing from that of LPS of Gram-negative aerobic bacilli where heptose is the basal sugar of the core (Lüderitz *et al.* 1966) and KDO provides the bridge between the polysaccharide and lipid moieties of the LPS (Osborn, 1963). Another rather unusual finding is the demonstration of fucose.

Apparently LPS of *Bacteroides fragilis* NCTC 9343 contains a wider range of sugar components than does LPS of *B. melaninogenicus* (Hofstad, 1968). However, further chemical studies have shown that the LPS preparations from *B. melaninogenicus* were heavily contaminated with intracellular glycogen (unpublished data), which may have masked the presence in acid hydrolysates of small amounts of other sugars.

Because of the low recovery and the limitations of the methods used, the quantitative analyses performed cannot give more than a rough estimation of the quantitative composition of the isolated LPS. However, the results are comparable with those obtained by quantitative analyses of LPS from other bacteria.

To our knowledge an amino sugar with properties similar to those of the unidentified amino compound has not previously been found in material isolated from bacteria. The chromatographic data obtained are not consistent with those reported for fucosamine (Crompton & Davies, 1958; Sharon, Shif & Zehavi, 1964) or for the fast-moving diamino-hexose isolated from *Bacillus subtilis* ATCC 9945 by Sharon & Jeanloz (1960). The possibility that the compound is part of a macromolecular structure other than LPS cannot as yet be excluded.

The *Bacteroides fragilis* LPS proved to be toxic for rabbits, but the endotoxic potency was low.

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