Volume 250, number 2, 565–569 FEB 07333 July 1989

Cleavage of the O antigen 4,5,12 of *Salmonella typhimurium* by hydrofluoric acid

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Received 4 May 1989

The effect of hydrofluoric acid (aqueous 48% HF) upon different lipopolysaccharides (LPS) was studied, employing conditions (48 h at +4°C) that are commonly used to dephosphorylate LPS. From the LPS of *Salmonella typhimurium* having the O antigen 4,5,12 almost all of the O-antigenic sugars (Abe, Gal, Glc, Man, Rha) were liberated in dialysable form, whereas the saccharide chains of *Salmonella* LPS with O antigen 6,7 (Man, Glc, GlcNAc) were resistant to HF. The lability towards HF was shown to be due to the presence of the deoxysugar *L*-rhamnose in the saccharide backbone of the O antigen 4,5,12, since only Rha was found as the terminal sugar in the corresponding dialysable material. Hydrofluoric acid can thus be used to specifically cleave Rha-containing polysaccharides.

Lipopolysaccharide; O antigen; Hydrofluoric acid; Rhamnose, L-; (*Salmonella typhimurium*)

1. INTRODUCTION

Lipopolysaccharides (LPS), also termed endotoxins, are complex macromolecules existing as structural components of the outer membrane of the Gram-negative bacterial cell envelope [1,2]. Chemically, they are composed of a lipid part termed lipid A, and a polysaccharide part which consists of two structurally distinct domains, the core oligosaccharide and the O-specific chain. Different bacterial genera and species elaborate lipopolysaccharides which are compositionally different. A characteristic feature of LPS is that they always contain phosphorus; this is found linked to both lipid A and to the core oligosaccharide region [1–3]. The detection and quantitation of 3-deoxy-D-*manno*-octulosonic acid (dOclA), which links the core oligosaccharide to lipid A, is hampered by phosphate substituents [4]. Furthermore, phosphate groups can complicate the methylation analysis of LPS-derived oligosaccharides, and the same holds true for studies of lipid A with laser desorption mass spectrometry [5,6]. To overcome these problems, chemical dephosphorylation of LPS by hydrofluoric acid is widely employed [4–8]. Typically a single treatment of LPS with aqueous HF brings about extensive dephosphorylation (>90%), whereas the macromolecular structure of LPS is preserved indicating that the ester and amide linkages of fatty acids in lipid A and interglycosidic linkages in the polysaccharide chain are resistant to hydrofluoric acid.

In studies with the O antigen 4,5,12-containing LPS of *Salmonella typhimurium* we found that besides dephosphorylation, hydrofluoric acid treatment caused a disruption of the LPS so that the O-antigenic sugars were almost completely liberated. It will be shown that the presence of the deoxysugar L-rhamnose in the O-antigen chain is responsible for this phenomenon.

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2. MATERIALS AND METHODS

2.1. *Bacterial strains and lipopolysaccharides*

Smooth *S. typhimurium* derivatives SH2183 [9] and SH5770 [10] with the O-antigenic formulae 4,5,12 and 6,7, respectively, were cultivated on L-agar [11] and the lipopolysaccharides ex-

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00145793/89/$3.50

tracted by phenol-water [12]. *S. typhimurium his-515* [13] with LPS of chemotype Ra (for description of LPS chemotypes see [1]) was cultivated as above, and LPS was extracted by the phenol/chloroform/petroleum ether method [14].

2.2. *Treatment of LPS with hydrofluoric acid*

Lipopolysaccharide (5–120 mg) was placed in a screw-stoppered 2-ml polypropylene tube (Nunc, Roskilde, Denmark) and 0.2–0.5 ml of precooled (+4°C) hydrofluoric acid (aqueous 48% HF; Merck, Darmstadt, FRG) was added. After keeping the suspension at +4°C for 48 h under stirring it was transferred into dialysis tubing (M_r cutoff 12000, Spectrum Medical Industries, Los Angeles, CA, USA) and dialysed (+4°C) against four 250-ml portions of distilled water. The contents of the dialysis tube (HF-LPS) was freeze-dried. A known amount of D-xylose (internal standard for neutral sugar analysis) was added to the combined outer dialysates, the solution neutralized (NH_4OH), reduced in volume, and freeze-dried. Below, all values for the amounts of components in the outer dialysates are adjusted to represent 1 mg of intact LPS.

2.3. *Analytical methods*

Gel filtration was performed using a Sephadex G-10 column (2.5 × 120 cm) with water as eluent, and 5-ml fractions were collected. The effluent was monitored at 206 nm and assayed for carbohydrate by the phenol-sulphuric acid reaction [15]. Total GlcN was analysed by the modified Morgan-Elson reaction [16] after hydrolysis of the sample in 4 M HCl (100°C, 16 h). The direct Morgan-Elson reaction (ME_{dir}) was done as above but without hydrolysis. Phosphate was determined according to [17]. The thiobarbituric acid assay [18] was carried out after hydrolysis of the sample in 0.1 M sodium acetate, pH 4.4 (100°C, 1 h [19]). Neutral sugars were analysed as their alditol acetates [20] by gas-liquid chromatography (GLC). Reductions were carried out using $NaBH_4$ or NaB^2H_4 (both from Merck). GLC of alditol acetates was performed with a Hewlett-Packard 5890A gas chromatograph (Avondale, PA, USA) equipped with a NB-225 fused silica capillary column (25 m × 0.32 mm i.d., Nordion Instruments, Helsinki, Finland). Temperatures of the injection port and the flame ionization detector were 260°C and 300°C, respectively. The temperature program was 200°C for 3 min, then 3°C/min up to 240°C. This program was also used for mass spectrometry, which was carried out with a combination of 5890 gas chromatograph (NB-225 column) and 5970 mass selective detector (Hewlett-Packard).

3. RESULTS AND DISCUSSION

Intact LPS of *S. typhimurium* SH2183 was found to contain GlcN, thiobarbituric acid-positive material (mainly dOclA), phosphate, and neutral sugars in the proportions shown in table 1. The O-specific side chain of *S. typhimurium* with the antigenic formula 4,5,12 LPS is built of repeating units consisting of a backbone trisaccharide (-2)D-Man(1-4)L-Rha(1-3)D-Gal(1-) to which abequose (Abe; 3,6-dideoxy-D-galactose) and to a lesser extent, D-Glc are attached as branches [21]. On the average SH2183 LPS contained six repeats of this oligosaccharide, since approximately six D-Man and L-Rha residues were found per mol of LPS. The lower proportion of Abe is attributable to its known instablity in neutral sugar analysis [22], whereas D-Glc and D-Gal are constituents of the core oligosaccharide as well. The reducing end of lipid A was quantitatively substituted, as indicated by the lack of reactivity in the direct Morgan-Elson assay.

Treatment of SH2183 LPS (8 mg) with aqueous 48% HF (48 h, +4°C) yielded materials termed HF-LPS (3 mg, nondialysable) and outer dialysate, respectively, the compositions of which are also shown in table 1. Compared to intact LPS, several differences were evident in the HF-LPS: (i) it was almost devoid of phosphate, (ii) it was positive (0.8 mol/mol LPS) in the direct Morgan-Elson assay, and (iii) it contained only minor amounts if any (Abe) of O-specific sugars. The average length of the O chain in HF-LPS was less than one repeat. The revelation of almost one mol of direct Morgan-Elson reactivity in parallel with the loss of phosphate is as expected since the position C-1 of the reducing GlcN in *S. typhimurium* lipid A is known to carry a phosphate substituent [3].

The materials lacking from the HF-LPS had been set free in dialysable form (table 1). The outer dialysate contained phosphate, O-specific sugars and some thiobarbituric acid-positive material which was obviously Abe, since it yielded an absorption spectrum with a maximum wavelength of 532 nm (cf. dOclA, 549 nm). Notably, L-*glycero*-D-*manno*-heptose (Hep) and 2-acetamido-2-deoxy-D-glucose (GlcNAc; table 1) were not liberated. An identical hydrofluoric acid treatment for an LPS lacking O side chains but having a complete core oligosaccharide (chemotype Ra; *his-515*) brought about no liberation of sugars (table 2). We conclude that except for dephosphorylation, the lipid A and the core oligosaccharide preserved their structure and macromolecular nature, whereas one or more linkages in the O-antigenic side chain were cleaved by hydrofluoric acid.

To find out whether the composition of the O-specific chain affects the degradability of LPS by hydrofluoric acid, we tested another *Salmonella* LPS with a different type of O-specific repeat with

Table 1

Chemical composition of intact *S. typhimurium* SH2183 LPS, hydrofluoric acid-treated LPS (HF-LPS) and the corresponding dialysable material (outer dialysate)

Component	LPS		HF-LPS		Outer dialysate (μmol/mg LPS)
	μmol/mg	mol/mol	μmol/mg	mol/mol	
D-Glucosamine	0.236	3.0[a]	0.450	3.0[a]	0.0
ME_{dir}	0.0	–	0.126	0.8	0.0
TBA-positive	0.137[b]	1.7	0.161[b]	1.1	+[c]
Phosphate	0.520	6.6	0.092	0.6	0.737
Abequose	0.279	3.5	<0.01	–	0.285
L-Rhamnose	0.476	6.0	0.108	0.7	0.242
D-Mannose	0.492	6.3	0.111	0.7	0.285
D-Galactose	0.698	8.9	0.568	3.8	0.346
D-Glucose	0.252	3.2	0.400	2.7	0.124
L-D-Heptose	0.226	2.9	0.431	2.9	0.0

[a] Assuming that 1 mol LPS contains 3.0 mol D-glucosamine
[b] Assuming the molecular mass of dOclA
[c] Present but not estimated; wavelength maximum 532 nm (most likely abequose)

the main chain structure Man-Man-Man-Man-GlcNAc (O-antigenic type 6,7 [23]). This LPS was not affected by hydrofluoric acid in its saccharide portion (table 2). Thus the interglycosidic linkages involving D-Gal, D-Glc, D-Man, GlcNAc, L-D-Hep and the ketosidic linkages involving dOclA were resistant to the treatment, whereas L-Rha in the O-specific chain emerged as a possible HF-sensitive candidate.

To investigate the site of cleavage in the O antigen 4,5,12, neutralised and concentrated outer dialysate resulting from hydrofluoric acid treatment of 120 mg of LPS was applied to a Sephadex G-10 column, from which two sugar-containing peaks appeared. The first one (pool A, 4 mg), eluting with the void volume contained D-Man, L-Rha, D-Gal, Abe, and D-Glc in a molar ratio of 0.8:0.4:1.0:0.1:0.1, respectively. The second peak (pool B, 20 mg), contained the same sugars in a molar ratio of 1.0:1.0:0.9:0.2:0.1. Aliquots of pools A and B were reduced with NaB^2H_4, hydrolysed (1 M CF_3COOH, 100°C, 1 h), reduced again ($NaBH_4$), per-*O*-acetylated, and analysed by GC-MS. In pool A, shifts of mass fragments indicating that reduction had occurred by NaB^2H_4 were present only in the mass spectrum of penta-*O*-acetyl-L-rhamnitol; accordingly it yielded prominent peaks at *m/z* 104, 116, 129, 146, 171, 188, 218 and 290. These were, however, always accompanied by peaks one mass unit lower, indicating that only partial reduction had taken place in the first step. Mass spectra of other peracetylated alditols (Abe, D-Gal, D-Glc, D-Man) yielded no ^2H-containing fragments. Pool B yielded penta-*O*-acetyl-L-rhamnitol whose mass spectrum was indistinguishable from that of NaB^2H_4-reduced and peracetylated L-Rha, whereas the other sugars exhibited no ^2H labelling. Thus the glycosidic linkages of L-Rha were selectively cleaved by hydrofluoric acid as illustrated in fig.1, leading to dialysable oligosaccharides with L-Rha as the terminal sugar. Pool B apparently represents Gal-

Table 2

Neutral sugar composition of fractions resulting from hydrofluoric acid treatment of LPS of *S. typhimurium his-515* (chemotype Ra) and SH5770 (O antigen type 6,7)

Component	Amount (μmol/mg LPS)			
	his-515		SH5770	
	HF-LPS	Outer dial.	HF-LPS	Outer dial.
D-Galactose	0.511	0.004	0.144	0.0
D-Glucose	0.597	0.019	0.172	0.0
D-Mannose	–	–	0.861	0.038
L-D-Heptose	0.597	0.0	0.170	0.0

Outer dial., outer dialysate

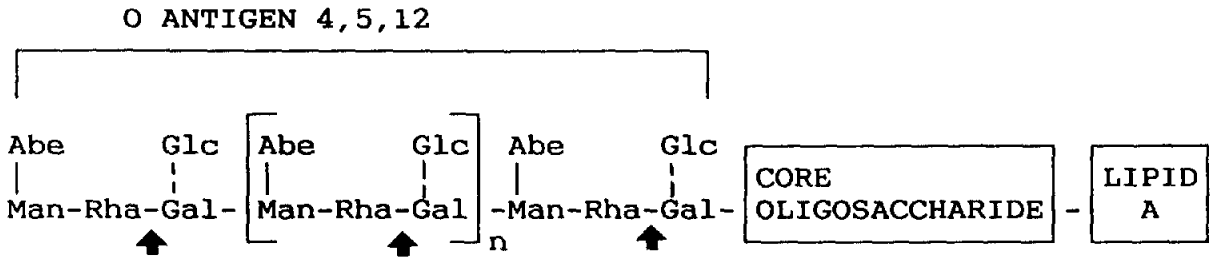

Fig.1. Sites of cleavage (arrows) of the O antigen 4,5,12 of *Salmonella typhimurium* LPS by hydrofluoric acid. The average value of *n* is 6 in the heterogenous LPS preparation. Broken lines indicate incomplete glucosylation, and in the intact LPS abequose (3,6-dideoxy-D-galactose) is partially *O*-acetylated at C-2.

Man-Rha trisaccharides with partial substitution by Abe and D-Glc whereas pool A contains larger oligosaccharides. These oligosaccharides are likely to be analogous to those produced from O antigen 4,5,12 by *endo*-rhamnosidases of bacteriophage origin [24,25]. The hydrofluoric acid treatment probably cleaved also a significant portion of the branch sugar (Abe, also a deoxysugar).

The glycosidic linkages provided by L-Rha in the O antigen are known to be susceptible to hydrolysis by 0.5 M H_2SO_4 at 100°C so that oligosaccharides with terminal L-Rha are produced; such treatments, however, also produce oligosaccharides stemming from the core [26,27]. It is apparent from the results that the ketosidic linkage provided by the deoxysugar dOclA, which is known to be highly labile towards acid hydrolysis by HCl or CH_3COOH [1,2], was resistant to hydrofluoric acid. To compare the effects of hydrofluoric acid and hydrochloric acid, we treated SH2183 LPS in analogous conditions with 37% HCl, and dialysed the mixture. Analyses of the compositions of the outer dialysate and HCl-LPS (not shown) revealed that in addition to the release of O-antigenic sugars, also Hep and dOclA were to a large extent released, indicating that the core oligosaccharide had also been partially cleaved. Furthermore, only little dephosphorylation was brought about by HCl.

To conclude, the remarkable sensitivity of Rha towards hydrofluoric acid must be taken into account when using HF with Rha-containing (lipo-) polysaccharides. On the other hand, HF enables the isolation of O antigen free from core sugars. It remains to be shown whether the high sensitivity to hydrofluoric acid is shared by other deoxysugars.

Acknowledgements: We thank P. Helena Mäkelä for critical reading of the manuscript, and Sirkku Waarala for skilful technical assistance.

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