STUDIES ON *O*-SPECIFIC POLYSACCHARIDES OF *SHIGELLA FLEXNERI* 1b(I) AND ITS SMOOTH MUTANT Z(III)

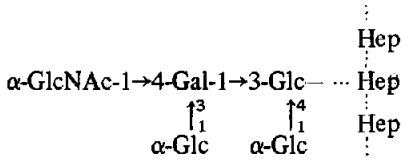
E.ROMANOWSKA and T.M.LACHOWICZ
Institute of Immunology and Experimental Therapy, Wrocław, Poland

Received 27 April 1970

1. Introduction

The *O*-antigen, a constitutive part of the cell walls of Gram-negative bacteria is a complex composed of *O*-specific polysaccharide and lipid. It has been shown (for review see [1a]) that the polysaccharide moiety of lipopolysaccharides consists of an inner core region (core polysaccharide) to which outer serologically specific polysaccharide chains (*O*-specific side chains) are attached.

Shigella flexneri O-antigens have been studied extensively by Simmons et al. [1–3]. These investigations elucidated the chemical composition of lipopolysaccharides of serotypes 1a, 2a, 3a, 4a, 5a, 6, variants X and Y, and the structure of the core polysaccharide characteristic for *Shigella flexneri*. They also yielded information on the structure of *O*-specific side chains of the serotypes mentioned above. According to these results, *Shigella flexneri* produces a cell wall lipopolysaccharide in which the core polysaccharide portion of the polymer has the following structure:



The *O*-specific portion of the polymer from *Shigella flexneri* is composed of a number of repeating units which form a primary unbranched chain of *N*-acetylglucosamine and rhamnose (molar proportion 1:2), substituted with secondary side chains of α-glucose. α-Glucosyl residues play an immunodominant role in antigenic factors I, II, IV, V and 7, 8. For the specificity of type antigen III, the important residue is *O*-acetyl-α-glucose.

It was observed that *Shigella flexneri* strains of serotype 1b (I, 3, 4, 6) split off the smooth mutant Z in which antigen type I is substituted for antigen type III, while group antigens 3, 4, 6 remain unaltered [4]. The mutant Z (III, 3, 4, 6) can be easily selected using one of the *Shigella flexneri* phages (F2) [5] or by serial passages of the original strain through white mice [6]. A similar alteration in antigen type of serotype 1b was also found in *lac*$^+$ recombinants obtained in crosses with *Escherichia coli* Hfr strains [7].

The present study was undertaken to explain the biochemical sense of the mutation described above. The chemical composition of the carbohydrate moiety of lipopolysaccharides of strain 1b(I) and its mutant Z(III) were defined. The lipopolysaccharides of R(rough)-forms of these two strains were also studied in this respect.

2. Materials and methods

Shigella flexneri strain 6713 of serotype 1b(I) (stock collection of our Institute) and its mutant Z(III) were used in the experiments. The growth of the bacteria in liquid medium and the phenol-water extraction of the dry bacterial mass [8] were performed as already described [9]. The purification of lipopolysaccharide (LPS) from phenol-water extracts was done by Sepharose 2B gel filtration [10].

Mild acid hydrolysis of LPS with 1% CH_3COOH (100°, 1.5 hr) to obtain the free polysaccharide and

North-Holland Publishing Company – Amsterdam

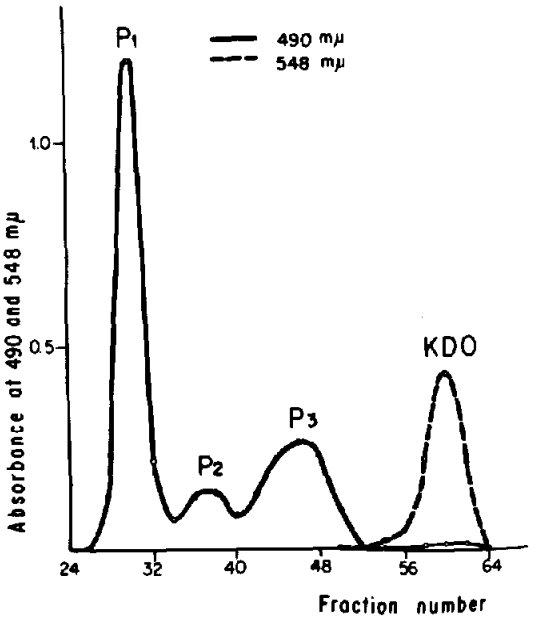


Fig. 1. Fractionation of polysaccharide 1b(I) (22 mg) on Sephadex G 50 (18 × 500 mm) equilibrated with pyridine-CH_3COOH buffer (pH 5.75, 10 ml of pyridine and 2 ml of acetic acid per liter); flow rate: 1.5 ml fraction per 30 min. Absorbance was measured at 490 nm for the phenol-sulphuric acid reaction (0.1 ml of fraction tested) and at 548 nm for the thiobarbituric acid reaction (0.2 ml of fraction tested).

the subsequent fractionation of the latter on Sephadex G 50 were performed as by Schmidt et al. [11].

Analytical determinations and paper chromatography of sugars were carried out as described [9, 10]. *O*-Acetyl groups were assayed according to Hestrin [12].

3. Results

During gel filtration on Sephadex G 50, the polysaccharide preparations obtained after CH_3COOH degradation of LPS 1b(I) and LPS Z(III) were separated into four fractions: P_1, P_2, P_3 and 2-keto-3-deoxyoctonic acid (KDO) (figs. 1 and 2). The analytical data for lyophilized P_1, P_2 and P_3 fractions are collected in table 1. For comparison, the sugar compositions of lipopolysaccharides isolated from 1b(I) strain, mutant Z(III) and their R-forms are also shown

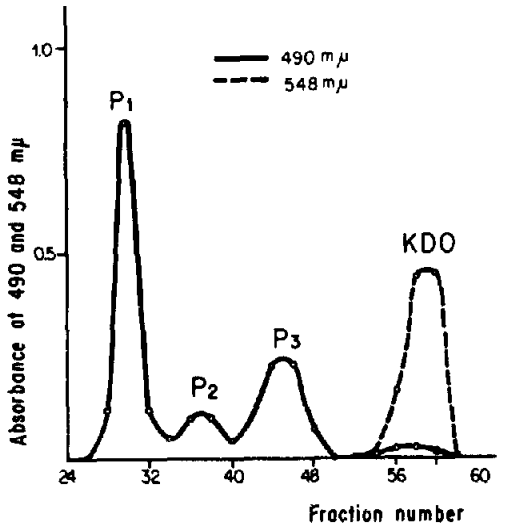


Fig. 2. Fractionation of polysaccharide Z(III) (19 mg) on Sephadex G 50. For details see the legend to fig. 1.

there. As may be seen, LPS 1b(I) and LPS Z(III) had the same sugar components: rhamnose, *N*-acetylglucosamine, glucose, galactose, heptose and KDO, with the difference that LPS Z(III) contained less glucose and more rhamnose than LPS 1b(I). Lipopolysaccharides from R-forms of 1b(I) strain and mutant Z(III) were almost free of rhamnose; their constituent glucose, galactose and heptose were in the approximate molar ratios, 3:1:2, in both lipopolysaccharides, giving evidence that they represent *Shigella flexneri* chemotype Ra of complete core structure [2].

Both P_1 fractions indicated *O*-specific side chain character, but their compositions were different. P_1 fraction of 1b(I) strain contained mainly glucose, *N*-acetylglucosamine, rhamnose and *O*-acetyl groups in the molar proportions 1.2 : 0.87 : 2.0 : 1.0, respectively. P_1 fraction of mutant Z(III) was almost devoid of glucose (the 2.5% of glucose constituted probably the contamination derived from core polysaccharide); this fraction was composed predominantly of *N*-acetylglucosamine, rhamnose and *O*-acetyl groups in the molar ratios 0.83 : 2.0 : 0 : 1.0, respectively.

P_3 fractions of 1b(I) strain and mutant Z(III) did not differ in their sugar composition. They consisted of *N*-acetylglucosamine, glucose, galactose, heptose,

Table 1
Sugar composition (in percentage *) of lipopolysaccharides and their polysaccharide fractions isolated from *Shigella flexneri* 1b(I), its smooth mutant Z(III) and their R-forms.

Components *	Lipopolysaccharides				Polysaccharide fractions					
	S-Forms		R-Forms		1b(I)			Z(III)		
	1b(I)	Z(III)	1b(I)	Z(III)	P_1	P_2	P_3	P_1	P_2	P_3
Rhamnose	17.2	24.2	⩽0.7	⩽1.2	32.0	30.5	3.4	58.3	31.8	3.6
N-Acetylglucosamine	14.6	16.2	11.2	11.1	19.2	14.2	9.9	32.5	14.8	9.8
Glucose	11.1	4.3	10.2	9.9	22.0	11.9	20.2	2.5	10.4	20.0
O-Acetyl groups	2.6	3.2	n.d.	n.d.	4.1	n.d.	n.d.	7.5	4.0	n.d.
Galactose	1.9	1.8	3.3	3.1	⩽0.8	n.d.	6.2	⩽0.8	n.d.	6.1
Heptose	3.4	3.0	8.4	8.1	1.6	5.6	14.8	1.4	n.d.	14.2
2-Keto-3-deoxyoctonic acid	4.2	4.0	8.0	7.7	0.3	0.7	2.8	0.4	0.9	3.1

*'%' = 100 (wt. of free sugar/wt. of polysaccharide analysed).
n.d. = not determined.

and KDO in the molar proportions 1.2 : 3.0 : 0.9 : 1.8 : 0.34, although some contamination by *O*-specific side chain component like rhamnose also occurred. Thus P_3 fractions appeared as polysaccharides of the chemotype Ra characteristic for *Shigella flexneri* complete basal structure.

P_2 fractions were obtained in very small quantities (less than 25% of the yield of P_1 fraction). They contained *O*-specific as well as core polysaccharide components. After rechromatography on Sephadex G 50, the P_2 fraction eluted at the same volume and its composition did not alter appreciably, indicating that it was not a mixture of P_1 and P_3 fractions. It seems probable that the P_2 fraction constitutes a product of breakage of LPS 1b(I) or LPS Z(III) in the external (*O*-specific) part so that shorter *O*-specific side chains and fragments of these chains attached to core polysaccharide are formed.

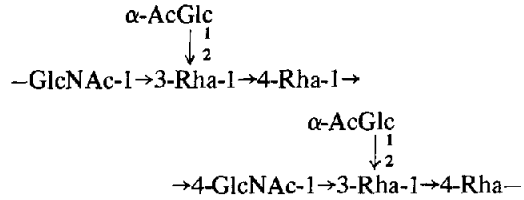
2-Keto-3-deoxyoctonic acid was identified in the last fraction eluted from Sephadex G 50. No other monosaccharides were found except a trace of rhamnose.

4. Discussion

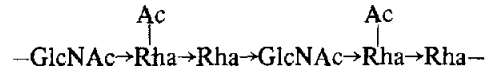
The repeating unit of the *O*-specific side chain of *Shigella flexneri* 1b(I) is composed of 1 molecule of glucose, 1 molecule of *N*-acetylglucosamine, 2 molecules of rhamnose and 1 *O*-acetyl group. The glucose probably forms α-glucosyl secondary side chains linked to carbon 4 of *N*-acetylglucosamine of the primary side chain, as in the 1a specific structure. Such a supposition is possible because the sequence α-glucosyl-1→4-*N*-acetylglucosamine is involved in antigen type I specificity, as proved by Simmons [3]. The repeating unit of the *O*-specific side chain of mutant Z(III) comprises *N*-acetylglucosamine, rhamnose and *O*-acetyl group in the molar proportions 1 : 2 : 1 respectively. As found in our study, LPS Z(III) is deficient in α-glucosyl secondary side chains, but this is not the case with the core structure of the polymer. Thus the mutation of serotype 1b(I) → Z(III) can be explained as a result of a defect in the specific UDP glucose transferase that is required to transfer the terminal α-glucosyl units to *N*-acetylglucosamine residues in antigen type I. A mutation of this kind is consistent with the biosynthetic pathway predicted by Simmons [3] for *Shigella flexneri O*-antigens.

On the other hand Z(III) antigen, in spite of its distinct type III specificity, does not contain the terminal *O*-acetyl-α-glucosyl residues which are found in serotype 3a antigen and are suggested to be involved in the antigenic factor type III [3]. In the light of our findings it seems that not only the *O*-acetyl-α-glucosyl-1→2-rhamnose configuration is characteristic for type III specificity, but also the rhamnose itself substituted with an *O*-acetyl group is sufficient to give rise to antigenic factor type III. To get the complete analogy between *O*-specific side chain structures of

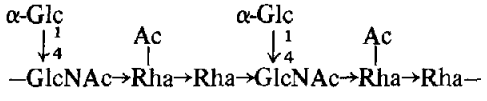
mutant Z(III) and serotype 3a it should be assumed that the same rhamnose of the primary side chain is substituted in both cases. The *O*-specific side chain of serotype 3a is, according to Simmons [3], the following:



Thus the structure of the *O*-specific side chain of mutant Z(III) seems to be:



As found in this study, the *O*-specific side chains of 1b(I) antigen were also *O*-acetylated. The rhamnose of *O*-antigen 1b(I) substituted with *O*-acetyl group is supposed to be the same as in *O*-antigen Z(III). The structure for 1b(I) *O*-specific side chain should be the following:



In spite of the *O*-acetyl-rhamnosyl configuration presumably present in LPS 1b(I), this antigen reveals no type III, but only type I specificity. This fact can be explained by the masking effect of the strong immunodominant-like α-glucosyl residue located on the adjacent 4-*O*-substituted *N*-acetylglucosamine. The serological evidence obtained in homologous complement fixation inhibition studies [13] confirmed the above supposition. With the systems: LPS 3a-type III antiserum and LPS Z(III)-type III antiserum, the P_1 fraction of mutant Z(III) was found to be a very effective inhibitor (80% and 100% inhibition respectively by 2 μg). With the same systems, P_1 fraction of serotype 1b(I) also inhibited, although to a smaller extent (about 30% inhibition by 5 μg). The cross reaction of P_1 fraction of serotype 1b(I) with type III system implies the presence of antigenic factor type III in serotype 1b(I). On the other hand no cross reaction between the P_1 fraction of mutant Z(III) and type I system was found.

The *O*-acetyl radicals in LPS 1b(I) and LPS Z(III) are rather stable. The molar ratio of *O*-acetyl to rhamnose amounts to 1:2 in the lipopolysaccharides as well as in the *O*-specific side chains (P_1 fractions) obtained after CH_3COOH hydrolysis.

Further experiments should be done to define exactly the structure of the primary side chain of serotype 1b(I) and its smooth mutant Z(III).

References

[1a] O.Lüderitz, K.Jann and R.Wheat, Comprehensive Biochem. 26A (1968) 105.
[1] D.A.R.Simmons, Biochem. J. 96 (1966) 903.
[2] J.H.Johnston, R.J.Johnston and D.A.R.Simmons, Biochem. J. 105 (1967) 79.
[3] D.A.R.Simmons, European J. Biochem. 11 (1969) 554.
[4] T.M.Lachowicz and M.Mulczyk, Arch. Immunol. Terapii Doświadczalnej 8 (1960) 437.
[5] S.Ślopek and M.Mulczyk, Arch. Immunol. Terapii Doświadczalnej 8 (1960) 417.
[6] M.Mulczyk, Arch. Immunol. Terapii Doświadczalnej 8 (1960) 1.
[7] T.M.Lachowicz, M.Mulczyk and U.Malerz, Arch. Immunol. Exptl. Therap. 14 (1966) 405.
[8] O.Westphal, O.Lüderitz and F.Bister, Z. Naturforsch. 7b (1952) 148.
[9] E.Romanowska and M.Mulczyk, European J. Biochem. 5 (1968) 109.
[10] E.Romanowska, Anal. Biochem., in press.
[11] G.Schmidt, B.Jann and K.Jann, European J. Biochem. 10 (1969) 501.
[12] S.Hestrin, J. Biol. Chem. 180 (1949) 249.
[13] E.Romanowska, E.Katzenellenbogen and C.Lugowski, unpublished observation.