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LIPID A MUTANTS OF VIBRIO CHOLERAE: ISOLATION AND PARTIAL CHARACTERIZATION

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Vibrio cholerae mutants resistant to common antibiotics and neutral and anionic detergents were isolated. Analysis of isolated outer membranes revealed a significant deficiency in the acylation of lipid A in the resistant strains. The content of amide-linked and ester-bound fatty acids in the lipid A of the mutant strains compared to that of the wild type was about 50-56% and 29-37% respectively. This defect was specific for lipid A as there was no change in the acylation of phospholipids. The reduction in fatty acid content of lipid A was reflected in the altered endotoxic properties in the mutant strains.

The amphiphilic macromolecule lipopolysaccharide (LPS) present in the outer membrane of gram negative bacteria is of considerable biomedical interest. The polysaccharide moiety of LPS which is relatively exposed in the outer membrane plays a crucial role in such diverse phenomena as antigenic specificity, permeability to exogenous compounds and binding of phages (1). The lipid A moiety of LPS is buried in the matrix of the outer membrane and has now been demonstrated to be the active component of endotoxin of gram negative bacteria (2,3). Importance of endotoxin is illustrated by the fact that even in nanogram amounts it is capable of eliciting a host of biological responses like pyrogenicity, hypotension, disseminated intravascular coagulation, localised Schwartzmann reaction etc. (3).

Isolation of mutants blocked at specific steps in LPS biosynthesis has played a key role in elucidation of the structure function relationship of the polysaccharide moiety of LPS (4). The biosynthesis of LPS is initiated from the lipid A moiety and as this region including the innermost core sugar KDO is

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Abbreviations

KDO : 3-deoxy-D-manno-2-octulosonic acid; **TLC** : Thin layer chromatography; **GLC** : Gas liquid chromatography; **SDS** : Sodium dodecyl sulphate; **PAGE** : Polyacrylamide gel electrophoresis; **BF_3** : Boron trifluoride.

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essential for cell survival, only a relatively few conditional lethal mutants defective in the synthesis of KDO which accumulate lipid A precursors have so far been reported (5). Obviously availability of mutants defective in lipid A biosynthesis would be of help in our understanding of the biological role of LPS.

The present study describes the isolation and preliminary characterization of two mutant strains of V. cholerae strain 569B which have specific blocks in the acylation of the glucosamine disaccharide of lipid A.

Materials and Methods

Organisms and growth media

The hypertoxinogenic strain 569B of V. cholerae carrying a streptomycin-resistant marker (50 ug/ml) was used as the parental strain. Cells were grown in DIFCO nutrient broth (NB) adjusted to pH 8.0 on a gyratory shaker at 37°C as described previously (6).

Isolation of mutants

For isolation of mutants, cells were mutagenised with N-nitroso-N'-nitro-N-methylguanidine (100 ug/ml, final) in 10mM phosphate buffer (pH 6.0) for 70 min at 25°C. The surviving cells were grown overnight and two classes of mutants were isolated by appropriate plating. The first class, designated lpa-1 was isolated from plates containing novobiocin (10 μg/ml), nalidixic acid (5 μg/ml) and rifampicin (0.5 μg/ml) while the second type designated lpa-2 was selected from plates containing SDS (0.05%) and Triton-X-100 (0.1%). Concentration of the inhibitory agents in the plates was at least 10 fold higher than the minimum growth inhibitory concentration of wild type cells. Both lpa-1 and lpa-2 maintained their resistance profile after repeated growth in control medium.

Isolation of membranes, LPS and lipid A

Crude cell membranes and outer membranes were isolated after mild sonication of cells suspended in 10mM HEPES buffer (pH 7.0) as described previously (6). LPS was extracted from crude membranes by hot phenol at 68°C (7) and purified by repeated centrifugation (105,000xg, 4 hrs, 10°C) in presence of 1mM $MgCl_2$. The final pellet was extracted 4 times with chloroform : methanol (2:1, v/v) to remove phospholipids. Contamination of protein in LPS was less than 2%. Lipid A was split off by treating LPS with 1% acetic acid at 100°C for 150 min. Preliminary experiments established that treatment with acetic acid for 150 min was both necessary and sufficient for quantitative release of lipid A. The insoluble lipid A was centrifuged off and the pellet dissolved in chloroform and the yield was assayed gravimetrically. The supernatant containing the liberated polysaccharide (PS) was dialysed extensively and lyophilised. Phospholipids were extracted from crude membranes by chloroform : methanol (2:1, v/v) (8).

Analysis of fatty acids

Total, ester and amide bound fatty acids of LPS were isolated as described by Rietschel et al (9) for assay by chemical methods. Ester bound fatty acids were assayed by the method of Snyder (10) while total and amide-bound acids by the method of Duncombe (11). For analyses by GLC, derivatisation of total fatty acids with 2M methanolic HCl or 14% BF_3/methanol, ester-bound fatty acids with 0.25M sodium methoxide and amide-bound fatty acids by treatment of de-O-acylated preparation with 14% BF_3/methanol were done as described by Mayer et al (12). The column used was a 5% SE-30 at 160°C using nitrogen as a carrier gas. For analysis of fatty acids in phospholipids, the crude membranes were extracted by chloroform : methanol (2:1, v/v) and the fatty acids assayed by GLC as described above for total fatty acids. Absence of 3-OH-tetradecanoic acid in such preparations ruled out contamination by LPS.

Assay of endotoxic activity

Standard methods were followed for assay of anticomplementary activity (13), localised Schwartzmann reaction and Limulus amoebocyte gelation (14).

Other analytical methods

Standard methods were followed for estimation of protein (15), total carbohydrate (16), sugars in LPS (12) and phospholipids (6). Glucosamine in lipid A was assayed (17) after removing the solvent under N_2 and hydrolysis with 4N HCl for 4 hrs. Phospholipid was quantitated from the content of bound phosphate (18). Outer membrane proteins were analysed on 12% SDS-PAGE according to the method of Laemmli (19). Assay of perosamine was done according to the method of Redmond (20).

Results

General properties of the mutant strains. Growth of the mutant strains lpa-1 and lpa-2 in NB and their sensitivity to vibrio specific group IV phage (21) were comparable with the parental strain. Resistant phenotypes of lpa-1 and lpa-2 to multiple antibiotics and detergents (used during their selection) could result from a change in the composition of the outer membrane. However, no significant difference in the profile of outer membrane proteins or in the phospholipid composition of outer membrane was seen between the wild type and mutant strains (data not shown). Moreover, the content of total carbohydrate, heptose, glucose and fructose in the polysaccharide moiety of LPS was similar in the wild type and mutant strains (Table 1). Perosamine, an O-antigenic sugar of *V. cholerae*, was detectable in all three strains. Although we did not assay perosamine quantitatively, the fact that proportion of the core sugars like heptose and glucose were the same in wild type and mutant strains suggested against any major change in the make up of the O-antigen of lpa-1 and lpa-2.

Analysis of lipid A. The amount of insoluble lipid A liberated during acetic acid treatment of LPS appeared to be much less from the LPS of the mutant strains and this visual impression was confirmed by a quantitative analysis of lipid A from the wild type and mutant strains. The yield of lipid A per mg of LPS in lpa-1 and lpa-2 was about 50% and 26% as compared to wild type strain 569B. Chemical assay of the total, amide and ester-bound fatty acids confirmed

Table 1. Composition of the polysaccharide moiety of lipopolysaccharide of wild type and mutant strains

Strain	Component analysed (μmoles/mg LPS)			
	Total carbohydrate	Glucose	Fructose	Heptose
Wild type	1.25	0.31	0.26	0.4
lpa-1	1.4	0.31	0.26	0.5
lpa-2	1.5	0.31	0.26	0.5

Components were assayed chemically as described in text.

Table 2. Lipid A composition of wild type and mutant strains

Strain	Yield of lipid A μg/mg LPS	μmoles/mg LPS				
		GlcN	PO_4	Total FA	Ester-bound FA	Amide-bound FA
Wild type	490	0.264	0.204	1.18	0.83	0.42
lpa-1	240	0.3	0.2	0.58	0.38	0.22
lpa-2	128	0.254	0.21	0.41	0.28	0.18

Chemical analysis carried out as described in text.

this deficiency in the acylation of lipid A in the mutant strains (Table 2). The amount of total fatty acids in 569B (1.18 μmole/mg LPS, Table 2) corresponded well with that reported previously (22). There was no change in the polar components, e.g. glucosamine or phosphate in the mutant strains (Table 2) indicating that the LPS molecule could be less hydrophobic in nature. The hydrophobicity of whole cells of the wild type, lpa-1 and lpa-2 were compared by partition experiments using xylene as the non-polar solvent (23). However, all three strains behaved similarly in this respect registering a loss of about 60% in turbidity after extraction of 2.0 ml cell suspension (in 0.85% NaCl) by 0.2 ml of xylene.

Table 3. Composition of fatty acids in lipid A of wild type and mutant strains

Fatty acid analysed	Total fatty acids			Ester-bound fatty acids			Amide-bound fatty acids		
	Wild type	lpa-1	lpa-2	Wild type	lpa-1	lpa-2	Wild type	lpa-1	lpa-2
3OH-10:0	0.90	0.50	0.45	0.40	0.15	0.12			
[a] Δ^2 12:1	0.30	0.16	0.15	0.60	0.27	0.13			
3-O-Me-12:0	n.d.	n.d.	n.d.	2.77	1.31	0.72			
3-OH-12:0	5.18	2.72	1.51	1.94	1.35	0.67			
14:1	3.74	1.89	1.18	3.44	1.66	1.04			
[a1] Δ^2-14:1	0.51	0.25	0.14	0.10	tr.	tr.			
3OH -14:0	3.59	1.91	0.80	n.d.	n.d.	n.d.	4.98	2.45	1.87
16:1	2.43	1.38	0.66	2.35	1.34	0.56			
16:0	3.42	2.06	0.76	2.88	1.95	0.92			
18:1	0.95	0.59	0.21	1.06	0.64	0.28			
18:0	0.56	0.47	0.12	0.39	0.40	0.18			
TOTAL	21.58	11.93	5.98	15.93	9.07	4.62	4.98	2.45	1.87

Values represent per cent fatty acids by wt. of LPS. n.d. : Not detected. a, a1 : artifacts of 3-OH dodecanoic and 3-OH tetradecanoic acids respectively produced during sample preparation.

Abbreviations : 3-OH-10:0, 3-hydroxydecanoic acid; Δ^2-12:1, dodecenoic acid; 3-O-Me-12:0, 3-acyloxyacyl derivative of dodecanoic acid (formed due to β-elimination, not detected in assay for total fatty acids); 3-OH-12:0, 3-hydroxydodecanoic acid; 14:0, tetradecanoic acid; Δ^2-14:1, tetradecenoic acid; 3-OH-14:0, 3-hydroxytetradecanoic acid; 16:1, hexadecenoic acid; 16:0, hexadecanoic acid; 18:1, octadecenoic acid; 18:0, octadecanoic acid.

Table 4. Endotoxicity of LPS isolated from wild type and mutant strains

Strain	Local Schwartzmann Reaction LPS (μg)				Limulus Amoebocyte Gelation LPS (μg)			
	50	20	10	5	10	1.0	0.1	0.01
Wild type	15mm	12mm	7mm	4mm	++++	++++	++++	+++
lpa-1	9mm	4mm	0	0	++++	+++	++	+
lpa-2	0	0	0	0	+++	++	+	0

Analysis of the fatty acids in the isolated lipid A by GLC confirmed the reduced acylation of lipid A in the mutant strains (Table 3). There was a general reduction in the level of both ester and amide-bound fatty acids. This reduction was more marked in the lpa-2 strain, the fatty acid content of 569B, lpa-1 and lpa-2 in lipid A being about 20%, 11% and 6% respectively (as % wt of LPS). The fatty acid profile of lipid A of 569B (Table 3) was similar to that reported previously (22).

To determine if the defect in acylation was specific for lipid A, the phospholipid from the crude membrane were extracted and analysed quantitatively for different phospholipids and the total fatty acids were analysed by GLC. No significant differences were seen in these parameters between 569B and the mutant strains indicating that defective acylation was restricted to lipid A.

Biological activity. LPS from both mutant strains exhibited markedly low endotoxicity in the localised Schwartzmann reaction. Thus, while no hemorrhagic lesion (whealing) was seen in lpa-2 even at the highest concentration of LPS tested (50 μg), the zone diameter with lpa-1 LPS was only 4 mm at 20 μg LPS and 9 mm at 50 μg of LPS compared to 12 mm and 15 mm for equivalent amounts of

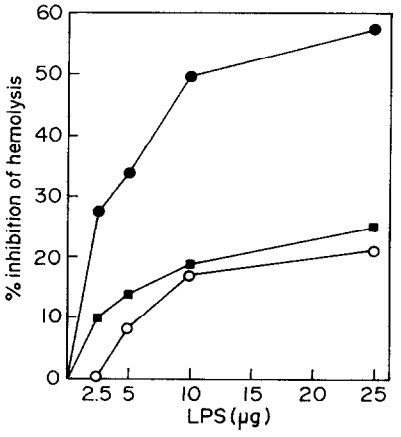

Fig. 1 : Anticomplementary activity of LPS of wild type (●) and mutant strains lpa-1 (■) and lpa-2 (o). Anticomplementary activity is expressed as per cent inhibition of hemolysis at different concentrations of LPS added to the undiluted serum (100-150 μl).

LPS from 569B (Table 4). In the limulus gelation assay, while the activity of the wild type LPS remained unchanged from 10 μg to 100 ng, the potency of lpa-1 LPS was approximately halved over the same range. The LPS of lpa-2 was even less active than the corresponding LPS from lpa-1 (Table 4). In the complement fixation assay at 2.5 μg LPS concentration, the per cent inhibition of hemolysis was around 30, 10 and 0 for 569B, lpa-1 and lpa-2 respectively (Fig. 1). This reduced complement fixing ability of the mutant strains was seen as the LPS concentration was increased and at 25 μg of LPS, the corresponding per cent inhibitions for the wild type, lpa-1 and lpa-2 were around 58, 25 and 20 respectively.

Discussion

Recent studies both with natural and synthetic preparations indicate that lipid A forms the bioactive centre of endotoxic properties exhibited by LPS of gram negative bacteria (2,3,14). In addition lipid A is involved in such important functions as maintaining the microviscocity and proper fluidity of the outer membranes (24). To date most of the mutants in lipid A synthesis seem to have defects in the polar substituents of lipid A (4,25) while conditional lethal mutants defective in synthesis of KDO have been shown to accumulate precursors of lipid A (3,4,5). The mutants lpa-1 and lpa-2 appear to have specific blocks in the acylation of lipid A and decrease in such acylation was nicely paralleled by a fall in endotoxicity (Tables 3 and 4, Fig. 1). The results clearly confirmed the role of fatty acids in the endotoxicity specially as the mutant lpa-2 which was less acylated than lpa-1 also exhibited the lowest biological activity in all the three assay methods used in the present study. It is interesting to note that even with such a serious defect in the acylation of lipid A (30% of wild type), the lpa-2 strain could grow and divide normally at 37°C. It would appear that the full complement of fatty acids in LPS may not be essential for survival under laboratory conditions. At the moment we cannot explain how resistance to detergents and hydrophobic compounds led to mutants defective in acylation of lipid A and work to clarify this is in progress.

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