Immunochemical Properties of the Lipopolysaccharide O-Antigen of *Vibrio cholerae* O1 in Relation to Its Chemical Structure

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D-Glucuronic acid and D-glucosamine have an immunodominant role in the lipopolysaccharide (LPS) O-antigen of both the Ogawa and the Inaba subtypes of *Vibrio cholerae* O1. This was evident from the pronounced inhibitory effect on the LPS precipitin reaction demonstrated by these monosaccharides and by oligosaccharides containing either of them which were isolated from LPS hydrolysate. There was a considerable decrease in the antibody-combining capacity of chemically modified LPS in which the carboxyl group of the glucuronic acid had been reduced. Similarly, on deamination, the O-specific polysaccharide fraction of the LPS molecule from both subtypes completely lost the ability to precipitate the LPS antibody.

INTRODUCTION

A partial chemical structure of the polysaccharide (PS) component of the purified O-antigenic lipopolysaccharide (LPS) fraction of *Vibrio cholerae* O1 has been established for both Inaba and Ogawa subtypes (Sen *et al.*, 1980; Majumdar *et al.*, 1983). On graded hydrolysis the PS from Ogawa and Inaba strains yielded three and five oligosaccharides respectively. The chemical structures of these eight oligosaccharides have been established. The work described here was aimed at elucidating the role of these oligosaccharides and their constituent monosaccharides as determinants of the immunochemical properties of the complete LPS antigen by observing (a) their inhibitory effect in the homologous LPS-anti-LPS precipitin system, and (b) the effect of modification of the chemical structure of the LPS on its precipitin property.

METHODS

Monosaccharides. These were procured from Sigma except for the heptoses, which were isolated from PS hydrolysate by preparative chromatography (Sen *et al.*, 1979).

Bacterial strains. Inaba 569B and Ogawa G2102, both *V. cholerae* O1 strains of classical biotype, were grown on nutrient agar in roll bottles for 18 h at 37 °C.

Preparation of LPS and PS. LPS was prepared after phenol treatment of live bacterial cells followed by fractional precipitation with cetyltrimethylammonium bromide as outlined previously (Guhathakurta & Dutta, 1974). The LPS fraction was finally purified by passing it through a column of Sephadex G-100, using 0.05 M-ammonium hydrogen carbonate for elution. Before any experiment the LPS preparations were confirmed to be free of protein, nucleic acid and degraded LPS by the biuret test, absorption at 260 nm and the gel diffusion test against bacterial antiserum respectively. Also, the LPS preparations from both Inaba and Ogawa strains, on being subjected to SDS-PAGE followed by silver staining (Tsai & Frasch, 1982), showed a single broad major band and a much fainter band co-migrating with the front. The LPS preparations were further subjected to high-voltage electrophoresis to

Abbreviations: LPS, lipopolysaccharide; PS, polysaccharide; CR-LPS, carboxyl-reduced lipopolysaccharide; OPS, O-specific polysaccharide; D-OPS, deaminated O-specific polysaccharide.
confirm that they moved as a single spot (Sen et al., 1979). PS was prepared from the purified LPS by treatment under nitrogen with 0.1 M-acetic acid for 2 h in a boiling water bath followed by extraction of the lipid with diethyl ether.

Preparation of O-specific polysaccharide (OPS) fraction. LPS from both the strains yielded OPS and core-polysaccharide on mild acid hydrolysis with 1% (v/v) acetic acid for 3 h at 105°C. The two fractions were separated on a Sephadex G-50 column (Majumdar et al., 1982). Both OPS and core polysaccharide gave a single spot when subjected to high-voltage electrophoresis but only OPS gave a single precipitin line in the gel diffusion test with antisera.

Preparation of oligosaccharides. Oligosaccharides from Inaba 569B were obtained by partial hydrolysis of the corresponding PS with 0.5 M-HCl for 2 h at 100°C followed by separation and purification by chromatography as described previously (Sen et al., 1980). For preparation of oligosaccharides from Ogawa G2102 the corresponding PS was first fractionated into OPS and core-polysaccharide. The Ogawa OPS was then treated similarly to the PS from Inaba 569B to yield the oligosaccharides (Majumdar et al., 1983).

Preparation of carboxyl-reduced LPS (CR-LPS). This was done by esterification of the carboxyl group of the uronic acid with 1-cyclohexyl-3-(2-morpholinoethyl) carbodi-imide-metho-p-toluene sulphonate followed by reduction with sodium borohydride (Majumdar et al., 1982). Uronic acid was not detected in the CR-LPS hydrolysate by paper chromatography. Colorimetric estimation of uronic acid in CR-LPS hydrolysate indicated 85-90% reduction.

Preparation of deaminated OPS (D-OPS). Ogawa and Inaba OPS were first N-deacetylated, followed by deamination with nitrous acid and reduction with sodium borohydride (Majumdar et al., 1983).

Preparation of antisera. Increasing doses (0.5, 0.75, 1.0, 1.25, 1.5 and 1.5 ml) of bacterial suspension (10⁸ c.f.u. ml⁻¹) were intravenously given twice a week to rabbits for three weeks. The animals were bled one week after the last injection (Guhathakurta & Dutta, 1974).

Quantitative precipitin reaction. The experiment was done with LPS, CR-LPS and D-OPS and rabbit antiserum. The reaction mixture contained 0.1 ml homologous antiserum, varying amounts of antigen (20-150 µg) and 0.15 M-phosphate-buffered saline pH 7.4, in a total volume of 0.55 ml. Appropriate blanks were set up. The mixtures were incubated for 1 h at 37°C followed by centrifugation at 4°C. The precipitates were collected by centrifugation at 4°C, washed twice with phosphate-buffered saline and dissolved in 2 ml 0.1 M-NaOH. The A₃₈₀ of each solution was measured.

Inhibition of quantitative precipitin reaction. The inhibitory effects of D-glucose, D-mannose, D-glucuronic acid (sodium salt), D-glucosamine hydrochloride, D-glycero-L-mannoheptose, L-glycero-D-glucoheptose and the oligosaccharides (as listed in Table 1) were tested. The compounds were added in increasing amounts to 0.1 ml portions of antiserum in duplicate and diluted with appropriate quantities of phosphate-buffered saline. The tubes were incubated for 1 h at 37°C. To each tube was then added a solution of LPS in phosphate-buffered saline (60 µg in each tube) to bring the system to equivalence. The pH value of the reaction mixture was confirmed (pH 7.4) to exclude any nonspecific inhibiting effect. The final volume in each tube was 0.55 ml. The precipitate was quantified as described before.

RESULTS

Inhibitory effect of monosaccharides on the immune precipitation of LPS

The constituent monosaccharides of the oligosaccharides isolated as products of the partial hydrolysis of OPS or PS from Ogawa and Inaba strains of V. cholerae, respectively, are indicated in Table 1. Inhibition of immune precipitation of the LPS from Inaba or Ogawa strains with homologous antisera in the presence of individual monosaccharides is presented in Table 2. Glucuronic acid was the most effective inhibitor, followed by glucosamine, irrespective of the subtype of the LPS. D-Glycero-L-mannoheptose was present only in the Inaba oligosaccharides; 2 µmol of this monosaccharide gave 20% inhibition of the homologous Inaba LPS precipitin reaction. The homologous Ogawa LPS precipitin reaction was inhibited by 8% by L-glycero-D-glucoheptose, which is present in the Ogawa oligosaccharides only. Inhibition by the heptoses was quantified only with 2 µmol amounts, due to their limited availability. Even at such a low concentration, the heptoses had considerable inhibitory effect on the precipitin reaction. Fig. 1 shows the effect of varying amounts of glucuronic acid and glucosamine on the immune precipitation of both Ogawa and Inaba LPS. Glucose and mannose had no effect on the precipitin system (Table 2).
Immunochemistry of O-antigen of V. cholerae O1

Table 1. Constituent monosaccharides of the oligosaccharides isolated after partial hydrolysis of LPS of V. cholerae subtypes Inaba and Ogawa

<table>
<thead>
<tr>
<th>Oligosaccharide</th>
<th>LPS source</th>
<th>Designation</th>
<th>Constituent monosaccharides</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inaba 569B</td>
<td>In-1</td>
<td>D-Glucuronic acid, D-glycero-L-mannoheptose</td>
<td></td>
</tr>
<tr>
<td></td>
<td>In-2</td>
<td>D-Glucose, D-glycero-L-mannoheptose</td>
<td></td>
</tr>
<tr>
<td></td>
<td>In-3</td>
<td>D-Glucose, D-glucosamine, D-glycero-L-mannoheptose</td>
<td></td>
</tr>
<tr>
<td></td>
<td>In-4</td>
<td>D-Mannose, D-glycero-L-mannoheptose</td>
<td></td>
</tr>
<tr>
<td></td>
<td>In-5</td>
<td>D-Mannose, D-glucuronic acid</td>
<td></td>
</tr>
<tr>
<td>Ogawa G2102</td>
<td>Og-1</td>
<td>D-Glucuronic acid, L-glycero-D-glucoheptose</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Og-2</td>
<td>D-Glucose, L-glycero-D-glucoheptose</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Og-3</td>
<td>D-Glucose, D-glucosamine, L-glycero-D-glucoheptose</td>
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</tr>
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</table>

Table 2. Inhibition by monosaccharides of precipitation of Inaba and Ogawa LPS with homologous antisera

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Amount added (μmol)</th>
<th>Antibody ppt.* (μg N)</th>
<th>Inhibition (%) ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Inaba</td>
<td>Ogawa</td>
</tr>
<tr>
<td>None</td>
<td></td>
<td>57</td>
<td>60</td>
</tr>
<tr>
<td>D-Glucose</td>
<td>20</td>
<td>57</td>
<td>59</td>
</tr>
<tr>
<td>D-Mannose</td>
<td>20</td>
<td>58</td>
<td>60</td>
</tr>
<tr>
<td>D-Glucuronic acid</td>
<td>20</td>
<td>13.5</td>
<td>27</td>
</tr>
<tr>
<td>D-Glucosamine</td>
<td>20</td>
<td>23</td>
<td>36.5</td>
</tr>
<tr>
<td>D-Glycero-L-mannoheptose</td>
<td>2</td>
<td>45</td>
<td>55</td>
</tr>
<tr>
<td>L-Glycero-D-glucoheptose</td>
<td>2</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* Means of five individual observations.

Table 3. Inhibition by their corresponding constituent oligosaccharides of precipitation of Inaba and Ogawa LPS with homologous antisera

<table>
<thead>
<tr>
<th>Oligosaccharide</th>
<th>Antibody ppt.* (μg N)</th>
<th>Inhibition (%) ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Inaba</td>
<td>Ogawa</td>
</tr>
<tr>
<td>None</td>
<td>57.0</td>
<td>60.0</td>
</tr>
<tr>
<td>In-1</td>
<td>36.0</td>
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</tr>
<tr>
<td>In-2</td>
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</tr>
<tr>
<td>In-3</td>
<td>28.5</td>
<td>-</td>
</tr>
<tr>
<td>In-4</td>
<td>51.0</td>
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<tr>
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<td>-</td>
<td>45.0</td>
</tr>
</tbody>
</table>

* Mean of five individual observations.

Inhibitory effect of oligosaccharides on the immune precipitation of LPS

Of the five oligosaccharides isolated from Inaba PS, 2 μmol, each of In-1, In-3 and In-5 inhibited the LPS precipitin reaction individually by 36%, 48% and 38% respectively (Table 3). In-2 and In-4 inhibited the precipitin reaction by only 9% and 10% respectively. Of the three oligosaccharides isolated from Ogawa OPS, Og-1 and Og-3, which contained glucuronic acid and glucosamine respectively in addition to L-glycero-D-glucoheptose, inhibited the immune precipitation by 34% and 26% respectively. Og-2, which contained neither glucuronic acid nor glucosamine, also inhibited (30%) the homologous precipitin reaction. The amount of the oligosaccharides required for effective inhibition was much less than that of the corresponding constituent monosaccharides. As such, the oligosaccharides In-1, In-3, In-5 and Og-1, Og-2,
Fig. 1. Effect of various concentrations of D-glucuronic acid and D-glucosamine on the immune precipitation of Ogawa LPS and Inaba LPS with homologous antisera. ○, Glucuronic acid with Inaba LPS; ●, glucuronic acid with Ogawa LPS; ▲, glucosamine with Inaba LPS; ■, glucosamine with Ogawa LPS. The points represent means of five determinations, with the range indicated by the bars (also applies to Figs 2-4).

Fig. 2. Effect of various concentrations of oligosaccharides, isolated from Inaba LPS, on the immune precipitation of Inaba LPS with homologous antisera. ●, In-3; ▲, In-5; ■, In-1; ○, In-4; △, In-2.

Fig. 3. Effect of various concentrations of oligosaccharides, isolated from Ogawa LPS, on the immune precipitation of Ogawa LPS with homologous antisera. ●, Og-1; ▲, Og-2; ■, Og-3.

Og-3 were better inhibitors than their constituent monosaccharides. Figs 2 and 3 demonstrate the effect of varying amounts of Inaba and Ogawa oligosaccharides as inhibitors in homologous precipitin systems.

**Effect of chemical modification of the LPS**

The immunodominant role of glucuronic acid and glucosamine was further confirmed with chemically modified LPS. We reported earlier that in CR-LPS the glucuronic acid is reduced to glucose, while other constituent sugars including glucosamine and heptoses remain unaffected (Sen et al., 1979). In the preparation of D-OPS, during deamination of the OPS the glucuronic
Irnmunochermistry of O-antigen of V. cholerae O1645

Fig. 4. Immune precipitation of LPS and CR-LPS, from Ogawa and Inaba subtypes, with homologous antisera. ●, Inaba LPS; ○, Inaba CR-LPS; ▲, Ogawa LPS; △, Ogawa CR-LPS.

acid is simultaneously destroyed. The heptoses, however, remain unaltered in D-OPS (Majumdar et al., 1983). On quantitative precipitation with homologous antisera both Ogawa and Inaba CR-LPS showed markedly decreased antibody-combining capacity (Fig. 4). D-OPS from both the strains gave no precipitation with their respective homologous antisera, indicating total loss of antibody-combining capacity due to removal of both glucuronic acid and glucosamine.

DISCUSSION

The present communication indicates that gluconic acid, glucosamine, and the two heptoses D-glycero-L-mannoheptose and L-glycero-D-glucoheptose were effective inhibitors of the V. cholerae LPS precipitin reaction. The most effective inhibitor oligosaccharides. viz. In-1, In-3, In-5 and Og-1, Og-3, all contain either glucuronic acid or glucosamine in addition to one of the two heptoses. The inhibitory effect demonstrated by Og-2 may be due to the presence of L-glycero-D-glucoheptose. The immunodominant role of gluconuronic acid and glucosamine in the O-antigen of V. cholerae O1 was further confirmed by the decreased antibody-combining capability of LPS when glucuronic acid was removed either singly (CR-LPS) or in combination with glucosamine (D-OPS). It may be noted that although glucosamine was identified as a constituent of some of the oligosaccharides (Table 1) it is possible that its amino group is acetylated in the purified V. cholerae LPS. The N-acetylglucosamine will be deacetylated during degradation of LPS by acid hydrolysis to oligosaccharides. N-Acetylglucosamine, however, did not inhibit the precipitation of LPS (Ogawa and Inaba) in homologous antisera.

The maximal inhibition produced by the monosaccharides, gluconic acid in particular, was considerably higher than that produced by the individual oligosaccharides containing them. This may be due to the presence of gluconic acid in more than one epitope of the complete antigen. It is possible that In-1 and In-5, both containing glucuronic acid, derive from different epitopes of Inaba LPS. Only one oligosaccharide containing glucuronic acid (Og-1) could be isolated from Ogawa OPS, however.

Isolation of the three oligosaccharides Og-1, Og-2 and Og-3 from the OPS of V. cholerae Ogawa G2102 demonstrates their occurrence in the O-specific region of the antigen. The Inaba oligosaccharides were prepared from PS, as the method for the separation of OPS and core region became known at a later date. Thus some of the Inaba oligosaccharides may have derived from the core region.

We reported earlier that the two epimeric heptoses D-glycero-L-mannoheptose and D-glycero-L-glucoheptose present in Inaba LPS are replaced by their corresponding optical isomers L-glycero-D-mannoheptose and L-glycero-D-glucoheptose in Ogawa LPS (Sen et al., 1979;
Majumdar et al., 1983). It is also interesting that the relative proportion of the two epimeric heptoses varies depending on the subtype of the LPS. In Inaba LPS the mannoheptose predominates over the glucoheptose whereas in Ogawa LPS the latter is the major heptose. The difference in the relative proportion of the heptoses between the Ogawa and Inaba LPS is reflected in the nature of the constituent heptose of the oligosaccharides from the two subtypes. It is possible that the heptoses D-glycero-L-mannoheptose and L-glycero-D-glucoheptose are involved in determining the subtype of *V. cholerae* O1 in conjunction with glucuronic acid and glucosamine. Raziuddin (1980) also hinted at the possible role of heptose and glucosamine in structure–function relationships of the PS moiety of *V. cholerae* O1. Clarification of the role of the heptoses as determinants of subtype specificity awaits further experimentation.

*V. cholerae* LPS is composed of lipid A, a core region and an O-specific side chain, similar to LPS from other Gram-negative bacteria. Kenne et al. (1979) proposed that the O-antigen of *V. cholerae* O1 from both Ogawa and Inaba LPS contains a homopolymer composed of a monomer unit of perosamine and 3-deoxy-L-glycerotetronic acid, where the C-3 position of the former and the C-2 position of the latter can be substituted. The homopolymer is not subtype-specific, being present in the LPS from both the subtypes. In view of the foregoing, it is probable that the immunodominant oligosaccharides were obtained as hydrolysis products from the substituent structures of the homopolymer at the positions indicated.

**REFERENCES**


