Studies on Heterogeneous Lipopolysaccharide Fractions of *Vibrio cholerae* 569B

By DEBOPAM CHAKRABARTI† AND ANADI N. CHATTERJEE*†

Department of Microbiology, Bose Institute, Calcutta 700 009, India

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By SDS-PAGE or gel filtration on Sephadex G-25, lipopolysaccharide (LPS) isolated from *Vibrio cholerae* 569B (Inaba) can be separated into two distinct fractions, one corresponding to smooth LPS and the other to rough LPS. Pulse-labelling of LPS with [14C]glucose showed that the rough form is synthesized first followed by the biosynthesis of the smooth form. A preferential release of the smooth LPS of *V. cholerae* 569B was also detected during normal growth of cells.

INTRODUCTION

Heterogeneity of LPS has been demonstrated in different Gram-negative bacteria (Alphen et al., 1983; Goldman & Leive, 1980; Jann et al., 1975; Koeltzow & Conrad, 1971; Palva & Mäkelä, 1980). The heterogeneous LPS fractions have been shown to be released in different proportions in the culture supernate when the organisms are grown in a liquid medium (Munford et al., 1980). We have found that the LPS of *Vibrio cholerae* 569B can be resolved into smooth and rough LPS forms upon gel electrophoresis. In the present paper we report on the rates of incorporation of radioactive glucose into the two LPS fractions and their release into the culture medium.

METHODS

_Bacterial strain and growth conditions._ *Vibrio cholerae* 569B (Inaba serotype) was obtained from the National Institute of Cholera and Enteric Diseases, Calcutta, India. It was grown at 37 °C in a gyratory shaker (New Brunswick Scientific Co.) in a medium (BP) containing 1% (w/v) beef extract (Centron Laboratories, India), 1% (w/v) peptone (Centron) and 1% (w/v) NaCl, pH 8.0.

_Pulse-labelling of LPS._ Exponentially growing 569B cells were pulse-labelled with 1.0 μCi [U-14C]-D-glucose ml⁻¹ [specific activity 114 μCi μmol⁻¹ (4.22 MBq μmol⁻¹)] in BP medium containing 1.4 × 10⁻⁶ M non-radioactive glucose for 15, 30 or 60 s. At specified time intervals incorporation was stopped by adding an equal volume of prewarmed 90% (w/v) phenol to the culture and the labelled LPS was isolated by the phenol/water extraction method (Westphal & Jann, 1965).

_Measurement of LPS release._ Labelled LPS was prepared by adding 0.5 μCi [U-14C]-D-glucose ml⁻¹ (specific activity 114 μCi μmol⁻¹) to an exponentially growing culture in BP medium containing 10⁻⁴ M non-radioactive glucose and the incorporation was continued for 90 min. Cells were then harvested (6000 g, 10 min at 25 °C), washed and re-suspended in prewarmed BP medium containing 1.6 × 10⁻² M non-radioactive glucose to an OD₂₅₀ of 0.15. Cells were allowed to grow with shaking in the non-radioactive medium for 180 min. The culture was then centrifuged (6000 g, 10 min at 25 °C) and the labelled LPS released in the supernate was isolated by phenol/water extraction (Westphal & Jann, 1965).

_SDSD-PAGE._ LPS was solubilized in sample buffer (Jann et al., 1975) and electrophoresed (5-6% gel) at 5 mA gel⁻¹ for 3-5 h. LPS bands on the gel were visualized by periodate-Schiff staining (Stewart-Tull, 1965; Dirienzo et al., 1978).

† Present address: Research and Development Division, Organon (India) Ltd, 182 A. J. C. Bose Road, Calcutta 700 014, India.
Determination of radioactivity in gel slices. After electrophoresis the entire gel was cut into 2 mm thick slices, which were put into scintillation vials containing 0.5 ml 1% (w/v) SDS and shaken for about 15 h at 37°C. Triton X-100-based scintillation fluid (Patterson & Greene, 1965) (5:0 ml) was added to each vial; the vials were kept in the dark for about 12 h before measuring the radioactivity in a Beckman LS-100C scintillation counter. The counting efficiency of 14C-labelled gels was about 50%.

RESULTS AND DISCUSSION

Incorporation of radioactive label in the heterogeneous LPS fractions

The LPS isolated from V. cholerae 569B can be separated into two bands (staining with periodate-Schiff reagent) in 5-6% gels with a relative mobility ($R_m$) of 0.6 (LPS I) and 0.88 (LPS II), respectively. Such heterogeneity of LPS has been demonstrated in Escherichia coli (Alphen et al., 1983; Goldman & Leive, 1980; McIntire et al., 1969; Morrison & Leive, 1975), Salmonella (Dirienzo et al., 1978; Goldman & Leive, 1980; Palva & Mäkelä, 1980; Ryan & Conrad, 1974), Citrobacter (Jann et al., 1975), Aerobacter aerogenes (Koeltzow & Conrad, 1971) and Alteromonas haloplanktis (Dirienzo et al., 1978). Chemical analysis of these LPS fractions indicated that LPS I corresponds to the smooth form of the LPS molecule containing both the core region and the O-antigenic chain while LPS II corresponds to the rough form containing only the core oligosaccharide. The polysaccharide moiety, obtained by hydrolysis of LPS by treatment with 1% (v/v) acetic acid for 150 min at 100°C, resolved into three peaks on Sephadex G-25 filtration. The first two peaks were polysaccharide in nature while the third peak contained only monomeric fructose. The composition and molar ratios of the monosaccharide present in the polysaccharide moiety, obtained by hydrolysis of LPS by treatment with periodate-Schiff reagent) in 5.6% gels with a relative mobility ($R_m$) of 0.6 (LPS I) and 0.88 (LPS II), respectively. Such heterogeneity of LPS has been demonstrated in Escherichia coli (Alphen et al., 1983; Goldman & Leive, 1980; McIntire et al., 1969; Morrison & Leive, 1975), Salmonella (Dirienzo et al., 1978; Goldman & Leive, 1980; Palva & Mäkelä, 1980; Ryan & Conrad, 1974), Citrobacter (Jann et al., 1975), Aerobacter aerogenes (Koeltzow & Conrad, 1971) and Alteromonas haloplanktis (Dirienzo et al., 1978). Chemical analysis of these LPS fractions indicated that LPS I corresponds to the smooth form of the LPS molecule containing both the core region and the O-antigenic chain while LPS II corresponds to the rough form containing only the core oligosaccharide. The polysaccharide moiety, obtained by hydrolysis of LPS by treatment with 1% (v/v) acetic acid for 150 min at 100°C, resolved into three peaks on Sephadex G-25 filtration. The first two peaks were polysaccharide in nature while the third peak contained only monomeric fructose. The composition and molar ratios of the monosaccharide present in the polysaccharide moiety, obtained by hydrolysis of LPS by treatment with periodate-Schiff reagent) in 5.6% gels with a relative mobility ($R_m$) of 0.6 (LPS I) and 0.88 (LPS II), respectively. Such heterogeneity of LPS has been demonstrated in Escherichia coli (Alphen et al., 1983; Goldman & Leive, 1980; McIntire et al., 1969; Morrison & Leive, 1975), Salmonella (Dirienzo et al., 1978; Goldman & Leive, 1980; Palva & Mäkelä, 1980; Ryan & Conrad, 1974), Citrobacter (Jann et al., 1975), Aerobacter aerogenes (Koeltzow & Conrad, 1971) and Alteromonas haloplanktis (Dirienzo et al., 1978). Chemical analysis of these LPS fractions indicated that LPS I corresponds to the smooth form of the LPS molecule containing both the core region and the O-antigenic side chain. While heptose, glucose and fructose were present exclusively in the core oligosaccharide, perosamine and rhamnose were present only in the O-antigenic regions.

To investigate the pattern of biosynthesis of the smooth and rough forms of LPS present in V. cholerae 569B, cells in the exponential phase of growth were pulse-labelled for 15, 30 and 60 s with [14C]glucose in the growth medium. The LPS extracted from cells labelled for 15 s had a peak of radioactivity corresponding to LPS II (Fig. 1). After 30 s of labelling the peak corresponding to LPS II increased while a small peak corresponding to LPS I was also detected. When the labelling was continued for 60 s the radioactivity recovered in the peaks corresponding to LPS I and LPS II was in the ratio 1:5:1:0. This is the normal distribution of total carbohydrate in the two LPS fractions as separated by SDS-PAGE. These results indicated that the label was first incorporated into the rough LPS II and the incorporation into the complete LPS I occurred subsequently. The labelling pattern is consistent with the known mechanism of LPS biosynthesis (Wright & Tipper, 1979), in which lipid A linked core oligosaccharide and O-side chain are synthesized independently and the complete LPS molecule is formed by the transfer of the complete O-side chain to the lipid A linked core oligosaccharide.

SDS-PAGE profile of the LPS released during normal growth of V. cholerae 569B

Gram-negative bacteria growing in liquid media release fragments of outer membrane (Hoekstra et al., 1976; Rothfield & Pearlman-Kothencz, 1969). Electron microscopic studies with V. cholerae cells also indicated formation of surface blebs which were released into the culture medium (Chatterjee & Chatterjee, 1974). Therefore it was of interest to determine if either of the two forms of LPS were preferentially released during growth of V. cholerae 569B. When [14C]glucose-labelled V. cholerae 569B cells were allowed to grow in a non-radioactive medium, most of the radioactivity recovered in the culture supernate was located in the LPS I fraction (Fig. 2). The ratio of total radioactivity in LPS I and LPS II was about 7:3:1:0. The same pattern was seen with cultures which were chased for 60 min and 120 min in the non-radioactive medium. This suggests a preferential release of smooth LPS molecules during growth. We have isolated a rough mutant strain (DB16) from 569B whose LPS resolved into a single band corresponding to LPS II on SDS-PAGE. As would be expected, the excretion of LPS from this mutant strain in the culture medium was reduced significantly. This preferential
Fig. 1. Kinetics of incorporation of radioactive label in LPS fractions. Cultures were pulse-labelled with $[^{14}C]$glucose and LPS was extracted by phenol/water and subjected to gel electrophoresis (see Methods). Gels were sliced (2 mm) and monitored for radioactivity. The origin is at the left-hand side. Time of pulse: 15 s (□), 30 s (△), 60 s (○).

Fig. 2. SDS-PAGE profile of LPS released in the culture medium. Exponentially growing V. cholerae 569B cells were labelled for 90 min with $[^{14}C]$glucose (see Methods) and grown in non-labelled medium. The LPS released into the culture supernate was extracted and subjected to SDS-PAGE. The origin is at the left-hand side.

release of smooth LPS from V. cholerae 569B is in agreement with recent studies in Salmonella typhimurium (Munford et al., 1980) where LPS excreted in the culture supernate was predominantly of the smooth form.

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