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Glycine-containing Lipopolysaccharide Isolated from *Vibrio cholerae* 4715 (NAG)

Morphological and chemical characterization of lipopolysaccharides (LPS) isolated from NAG (non-agglutinable) type *Vibrio cholerae* was carried out and glycine was found in a significant amount in lipid A fraction of LPS. Some distinct differences from cholera vibrios in the chemical composition were revealed.

Keywords——lipopolysaccharide; LPS; *Vibrio cholerae*; NAG vibrio; glycine

Based on study and subsequent proposal by Sakazaki and his colleagues,[1,2] so-called NAG vibrios are now included taxonomically into *Vibrio cholerae*. NAG vibrios, not agglutinable with diagnostic antisera which recognize the classical and *El Tor* cholera vibrios have now gained a new inportance in recent years. While cholera vibrios (classical and *El Tor*) have historically been associated with epidemic cholera, NAG vibrios, once excluded from the consideration for cholera epidemiology, have now been attracting many attentions because of their pathogenicity in producing diarrheal disease that is identical or very similar, clinically, to cholera.[3,4]

The differenciation of NAG vibrios in agglutination from the cholera vibrios is based on their heat stable somatic or 0 antigen, *i.e.*, endotoxic lipopolysaccharides (LPS). However, very little has been known of LPS of NAG type *V. cholerae*. To our best knowledge, only a brief analytical data were presented by Guhathakurta and Dutta[5] for the gross contents of sugars, but not for the individual monosaccharide constituents present in LPS.

Morphological and more penetrating chemical characterization of LPS isolated from NAG type *Vibrio cholerae* has been done in the present study, and glycine has been found in a significant amount in lipid A fraction of LPS. Some distinct differences in the chemical composition between the cholera vibrios (classical and *El Tor*) and NAG vibrios were revealed.

V. cholerae 4715 (NAG, 03 serotype, according to Sakazaki and Tamura[6]) was employed. Microbes were grown in the GYP medium (glucose 1%, yeast extract 0.5%, peptone 1%, pH 8.0) at 37° for 16 hr under shaking. The cells were disintegrated using Bühler's cell homogenizer (Edmund Bühler, Tübingen, West Germany) with glass beads (0.11—0.12 mm in diameter) as described in our previous paper,[7] and crude cell walls were isolated from the disintegrated cells by differential centrifugation. LPS were isolated from either acetone-dried or lyophilized cell walls according to Westphal's phenol-water technique[8] and highly purified by repeated centrifugation and RNase treatment. Lipid A and degraded polysaccharide were isolated by treatment of LPS with 5% acetic acid at 100° for 2 hr according to Galanos *et al.*[9]

Specimens for electron microscopy were negatively stained with 1% ammonium molybdate adjusted to pH 7.0 and examined with a Hitachi Model 11E electron microscope at 75 kV.

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Quantitative and qualitative analyses were performed as follows: Total phosphorus, reducing sugar activity, and total amino sugar were determined according to the methods described in our previous paper.[10] Amino acids and amino sugars were determined by JLC-6AS amino acid autoanalyzer (JEOL) after hydrolysis in 6 N HCl at 110° for 16 hr and 4 N HCl at 100° for 8 hr, respectively. Neutral sugars were determined after hydrolysis in 2 N trifluoroacetic acid at 120° for 1 hr as alditol acetates by gas-liquid chromatography (column: 3% ECNSS–M coated on Chromosorb W, 2 m×3 mm) according to Laine *et al.*[11] Fatty acids were determined by gas–liquid chromatography (column: 3% OV-1 or 25% DEGS coated on Chromosorb W 2 m×4 mm) of the methyl esters prepared according to Hoshi *et al.*,[12] after hydrolysis in 4 N HCl at 100° for 4 hr. Identity of the sugars and fatty acids was based on retention time values compared to those of authentic standards. Heptose was determined by either gas–liquid chromatography or colorimetry,[13] KDO (2-keto-3-deoxyoctonic acid) according to Osborn,[13] and protein according to Lowry.[14]

Highly purified LPS, when under an electron microscope observed, revealed a remarkably homogeneous filament structure like a ribbon and net-like aggregates. Diameter of the filaments was 20—23 nm.

The overall chemical composition of LPS was as follows: Protein, 2.1%; reducing sugar, 36.9%; amino sugar, 15.0%; total phosphorus, 2.3%; total lipid, 26.6%. Total recovery was 67.9%. Sugar components detected were glucose 4.9%, galactose 1.4%, glucosamine 5.2%, and an unidentified amino sugar, 4.7% as glucosamine. In addition to those sugar components, two kinds of heptose were detected. One was identified as L-glycero-D-mannoheptose, a typical component sugar of LPS of *Salmonella* and many of other related bacteria. The other, which was eluted just in front of L-glycero-D-mannoheptose in gas–liquid chromatography, was tentatively identified as D-glycero-D-mannoheptose since analysis by gas chromatography mass spectrometry of the alditol acetate derivatives of the two heptoses afforded an identical mass spectrometric fragmentation pattern. The contents of these were 5.4% for L-glycero-D-mannoheptose and 6.3% for D-glycero-D-mannoheptose. So far we have investigated, D-glycero-D-mannoheptose have not been detected as a constituent of LPS of O group I cholera vibrios such as *V. cholerae* 35A3 (Inaba), 569B (Inaba), NIH 41 (Ogawa), NIH 90 (Ogawa) and P1418 (Ogawa). Quite interestingly, no evidence was obtained for the presence of KDO, another usual component sugar other than L-glycero-D-mannoheptose of LPS in gram-negative bacteria, in either gas–liquid chromatographic or colorimetric analysis of the hydrolysates (0.02 N H_2SO_4, 20 min at 100°) of the LPS. This result was partly similar to that of Jackson and Redmond[15] with LPS from *V. cholerae* 569B (Inaba) where not only KDO but also galactose was absent. With regard to the absence of KDO in LPS, we have confirmed that *V. cholerae* 35A3 (Inaba), NIH 41 (Ogawa), and NIH 90 (Ogawa), in addition to *V. cholerae* 4715 (NAG), lack KDO as well as galactose in their LPS. Although LPS of *V. cholerae* 4715 (NAG) lack only KDO, and not galactose, it is considered that the lack of KDO in LPS may be of taxonomic significance as one of characteristics of *Vibrio cholerae*. The most striking difference in the chemical composition between LPS of NAG vibrios and of the cholera vibrios was that NAG LPS were entirely devoid of quinovosamine (2,6-dideoxy-2-aminoglucose), an characteristic amino sugar component isolated and identified by Jann *et al.*[16] from LPS of *V. cholerae* 569B (Inaba) and NIH 41 (Ogawa), and also detected by Hisatsune and Kondo[17] in LPS from *V.*

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cholerae 35A3 (Inaba), NIH 90 (Ogawa) and P1418 (Ogawa) as well as 569B (Inaba) and NIH 41 (Ogawa). An unidentified amino sugar which was detected in NAG LPS showed a quite different behaviors from those of quinovosamine in thin-layer chromatography and gas-liquid chromatography.

Table I. Amino Acids Present in LPS Isolated from *Vibrio cholerae* 4715 (NAG)

Amino acid	nmol/mg LPS	Amino acid	nmol/mg LPS
Asparatic acid	5.9	Glycine	151
Threonine	2.0	Alanine	5.6
Serine	3.2	Glucosamine	241
Glutamic acid	3.3		

Amino acid composition (Table I) revealed that only a limited number of amino acids were found and their amount was very low except for glycine. Of particular interest was that as much as 26—75 times more glycine than other individual amino acids was present in LPS and, furthermore, a larger content of glycine was found exclusively in lipid A fraction and not in degraded polysaccharide fraction of LPS. The molar ratio of glycine to glucosamine was 1: 1.78 in LPS and 1: 2.38 in lipid A fraction. Amino acids were hitherto only rarely reported as constituents of O-antigens of gram-negative bacteria. An alkali-labile glycine residue was reported to be present in the lipopolysaccharides (LPS) isolated from an *Escherichia coli* serotype by Gentner and Berg,[18] and a substantial amount of lysine in LPS from *Proteus mirabilis* 1958 by Kotelko *et al.*[19] and by Gromska and Mayer,[20] and amide-linked L-alanine in *Proteus mirabilis* 19ϕ by Gmeiner.[21] While methionine and glycine were reported to be present also in LPS of *V. cholerae* 569B (Inaba), no quantitative data for them were presented.[15] In our analysis of LPS of *V. cholerae* 569B, strains of which was derived from Dr. Finkelstein's laboratory, the presence of methionine and glycine was also observed among the other amino acids in the acid hydrolysates of LPS, but no marked difference was recognized in the amounts of methionine and glycine and those of other individual amino acids.

The fatty acid composition revealed that the nonhydroxy-fatty acid fraction of the lipid of LPS consisted predominantly of 39.9% of tetradecanoic and 46.6% of hexadecanoic acids and the 3-hydroxy-fatty acid fraction, 48.0% of 3-hydroxy-dodecanoic and 50.2% of tetradecanoic acids.

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