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Critical Aggregation Concentrations of Gram-Negative Bacterial Lipopolysaccharides (LPS)

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Bacterial endotoxins or lipopolysaccharides (LPS), cell wall components of gram negative bacteria, are involved in septic shock. While the carbohydrate structure of LPS have been studied in the past, little is known about the macromolecular structure and formation of LPS fragments in blood. It is believed that amphiphilic molecules such as LPS occur as monomers and aggregate into macromolecular structures above a critical micelle or critical aggregate concentration, CAC. The CAC of Lipid A, a LPS precursor, and several LPS serotypes of varying molecular weight and different polysaccharide chain lengths were established by static light scattering and by steady-state fluorescence spectroscopy by incorporation of the fluorescent probe, NPN, N-phenyl-1-naphthylamine. The CAC for short polysaccharide chain mutant LPS *S.minnesota* Re 595 (MW 2,500) and Lipid A from *S.minnesota* Re 595 (MW 2000) were 4 μM and 5 μM respectively. The CAC of LPS from heterogenous long O-antigen polysaccharide chain bacterial serotypes: *S.minnesota wildtype* were 11 μg LPS/ml, *S.typhimurium* 14 μg LPS/ml and *E.coli* 0111:B4 22 μg LPS/ml, respectively. The result obtained suggests that critical aggregate concentration and solubility of LPS is a function of polysaccharide chain length.

Key Words: **critical aggregate concentration; CMC; fluorescence; lipid A; LPS particles; supramolecular assembly.**

Bacterial endotoxins or lipopolysaccharides (LPS) are outer cell wall products from gram-negative bacteria that initiate septic shock in animals and humans (1) through an up-regulated host immune response. Early detection of bacteria or other infectious material is important for patient survival.

Bacterial LPS are amphiphilic molecules, and are heterogenous both in size and composition. LPS are composed of three parts (Fig. 1): a negatively charged lipid A group with six or seven hydrocarbon chains attached to a non-reducing, biphosphorylated, glucosamine disaccharide head group, that anchors the LPS to the outer monoleaflet of the bacterial outer membrane; a core oligosaccharide covalently bound to the lipid A group via 3-deoxy-D-manno-octulosonic acid (KDO); and, the O-antigen polysaccharide chain which protrudes into the surrounding medium (2, 1, 3). The carbohydrate cores and the O-antigen chain vary both in composition and in the number of polysaccharide repeat units for different bacterial serotypes but the mechanisms for the differing toxicity between serotypes have not been fully resolved. Recent findings suggest that LPS lacking the O-antigen induce pro-inflammatory cytokine response in macrophages at a higher incidence compared to LPS having the O-antigen (4).

In the host during infection a lipopolysaccharide binding protein, LBP, always present in the blood, has been shown to interact with the negatively charged lipid A portion of the LPS (5). The lipid A moiety of the LPS molecule excerpts endotoxic effect on cells (6). Lipid A subunits from different bacterial strains have similar chemical composition and synthetic lipid A has been shown to retain endotoxicity (7). Despite extensive research on LPS immunobiology (7–12) the physical and structural organization of LPS aggregates in the blood and their involvement with septic shock syndrome is yet to be determined. It is not known if LPS molecules interact with LBP as either LPS monomers, fragments or aggregates,

$$\text{MONOMER} \Leftrightarrow \text{FRAGMENT} \Leftrightarrow \text{AGGREGATE}$$

and if any of the molecular organization of LPS in solution or blood is a reversible, concentration or reaction rate limited process. It is suggested, that LPS

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Abbreviations: CMC, critical micelle concentration; CAC, critical aggregate concentration; LBP, lipopolysaccharide binding protein; LPS, lipopolysaccharide; NPN, N-phenyl-1-naphthylamine; SBP, sulfobetaine palmitate (N-hexadecyl, N-dimethyl-3-amino-1-propane sulfonate); TRIS (Trizma), 2-amino-2-(hydroxymethyl-1,3-propanediol).

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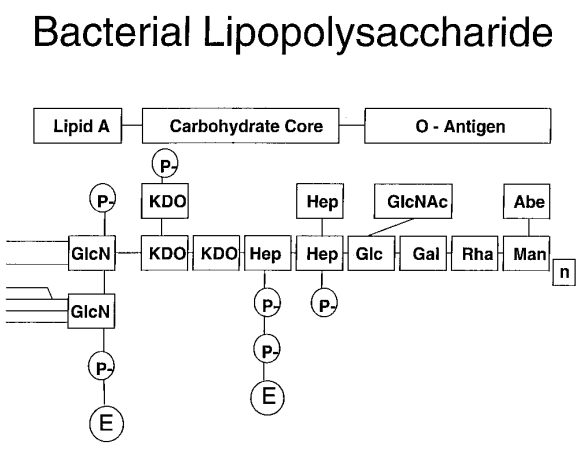

FIG. 1. Schematic LPS structure.

molecules form multimeric clusters (13) above a certain concentration in aqueous environments. This work proposes a convenient system for characterizing the critical aggregate concentration, CAC, to characterize the physical organization of large amphiphilic molecules such as LPS and other macromolecular amphiphilic structures. We have earlier (14) analyzed fluorescent labeled LPS from several bacterial species inserted into a detergent micelle in fluorescence resonance energy transfer experiments, FRET, where we determined the extended length of several LPS serotypes in solution. In this study we use steady-state fluorescence spectroscopy together with N-phenyl-1-naphthylamine (NPN) as a fluorescent marker, well known for its propensity to partition into hydrophobic environments, to characterize the CAC of several LPS serotypes in solution.

MATERIALS AND METHODS

Materials. The following chemicals and reagents were purchased from Sigma Chemical (St. Louis, MO): Lyophilized lipopolysaccharides (*E.coli* 0128:B12, *E.coli* 0111:B4, *E.coli* 055:B5, *E.coli* 026:B6, *S.minnesota* Re595, *S.minnesota wildtype, S.typhimurium, S.abortus equi, Se.marcescens*) and Lipid A from LPS *S.minnesota* Re595, TRIS (Trizma) preset crystals (TRIS, 2-amino-2-(hydroxymethyl-1,3-propanediol and TRIS hydrochloride), sodium chloride (NaCl), sulfobetaine palmitate (SBP, N-hexadecyl-N,N-dimethyl-3-amino-1-propane sulfonate). N-phenyl-1-naphthylamine (NPN) was obtained from Molecular Probes (Eugene, OR).

Sample preparation. All freeze-dried LPS were rehydrated in a dilute salt buffer solution containing 0.02 M TRIS and 0.15 M NaCl at pH 7.5 and used without further purification. The buffer solution was filtered through a Supor-200 (Gelman Sciences, Ann Arbor, MI), 0.2 μm filter and degassed before use. It is considered that dilute salt solutions < 0.3 M do not decrease solubility or have profound hydrophobic effects of amphiphiles (15).

As a control for the fluorescent NPN assay a stock solution of 0.1 M SBP (detergent), was prepared in distilled water. Control CMC experiments using SBP were performed in the salt buffer.

Surfactant CAC/CMC determination using fluorescence spectroscopy. The CAC/CMS of the LPS and control detergents SBP were determined using a protocol modified from Brito and Vaz (16). We used a buffer containing 0.02 M TRIS, 0.15 M NaCl at pH 7.5. As the NPN molecule partitions into the hydrophobic compartment of the LPS aggregate or detergent micelle its emission peak shifts from about 475 nm to 425 nm and the quantum yield increases. The increase in fluorescence was measured for 5 μM NPN (LPS and SBP varying from 0 to 100 and 200 μM, respectively). The samples, were incubated for 30 min. at 25°C, prior to fluorescence measurements, performed at 25°C, with excitation wavelength at 350 nm, emission at 425 nm with slits set at 1.7 nm resolution in a Spex Fluorolog 112 -1680 (Spex Industries Inc., Edison, NJ) equipped with a Hamamatsu R928 PMT. Disposable acrylic cuvettes (VWR Scientific) with 1 cm path length were used for all experiments.

RESULTS

Critical aggregate concentration of short chain bacterial LPS. Steady-state fluorescence spectroscopy measurements of NPN intensity with increasing LPS concentration indicate that at a specific LPS concentration in the solution the NPN intensity increase dramatically. The change of fluorescence intensity depends on the preferential partitioning of NPN molecules into the hydrophobic core of LPS aggregates.

The CAC for LPS aggregates from *S.minnesota* Re 595 and lipid A from *S.minnesota* Re 595 were determined by the relative NPN fluorescence (Fig. 2). The short chain LPS serotype lacks both the O-antigen chain as well as the KDO-moeity. Both LPS and lipid A have very similar CAC, 4 μM and 5 μM, respectively. The CAC is found at the point where the two slopes of the curve intersect. Similarly, the CAC for the same LPS serotype, as above, and lipid A were examined by static light scattering. As the LPS aggregate into macromolecular structures at increased concentration they

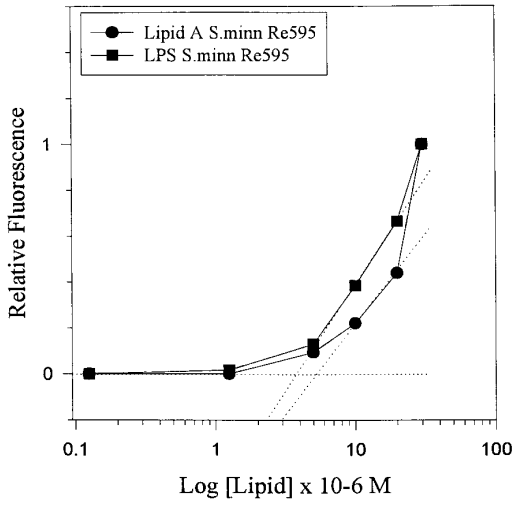

FIG. 2. CAC determination for short chain LPS serotypes. Relative fluorescence intensity of 5 μM NPN, at 425 nm as a function of concentration for LPS and lipid A from *S.minnesota* Re 595 at 20°C, in 0.02 M TRIS, 0.15 M NaCl at pH 7.5. The NPN was excited at 350 nm and the fluorescence peak was normalized to 1.0. The intensity of NPN increases sharply in the range of CAC. The actual CAC can be determined where the lines of the two slopes of each curve intercept.

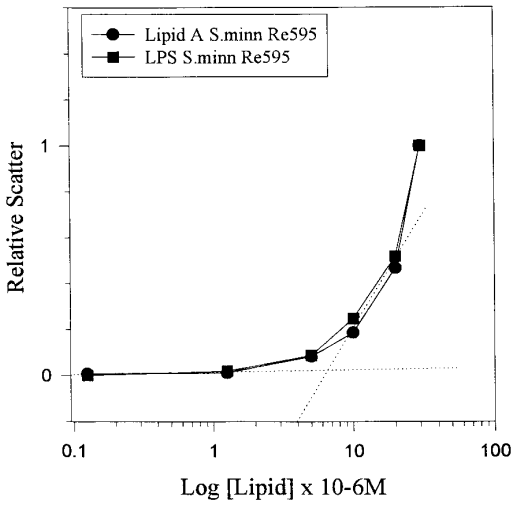

FIG. 3. CAC determination for short chain LPS serotypes. Relative light scatter, at 90°C as a function of concentration for LPS and lipid A from *S.minnesota* Re 595 at 20°C, in 0.02 M TRIS, 0.15 M NaCl at pH 7.5. Excitation and emission set at 460 nm. The light scatter intensity of LPS and lipid A increases sharply in the range of CAC. The LPS the peak intensity was normalized to 1.0. The CAC can be found where the lines of two slopes intercept.

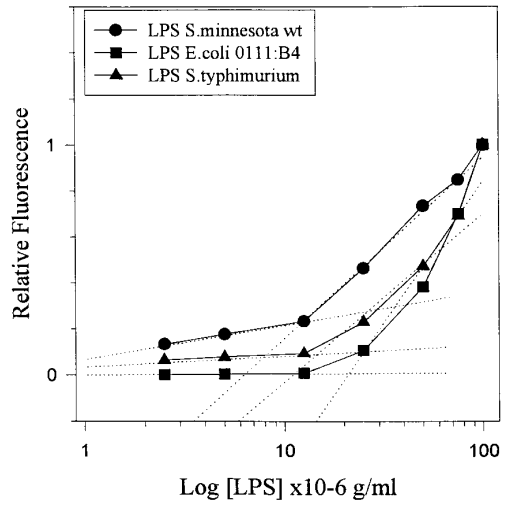

FIG. 4. CAC detion for several LPS serotypes, at 20°C, in 0.02 M TRIS, 0.15 M NaCl at pH 7.5. The NPN was excited at 350 nm and the fluorescence peak was normalized to 1.0. n= average of 3 experiments. The relative intensity of NPN increases sharply in the range of CAC. The CAC can be found where the lines of the two slopes of each curve intercept.

scatter light. The CAC were determined as above and were found to be at low ?M concentrations, 6 μM and 7 μM respectively (Fig. 3).

Long chain heterogenous bacterial LPS fragments. Figure 4 shows CAC determinations of LPS incorporated into LPS aggregates from *S.minnesota wildtype, E.coli* 0111:B4 and *S.typhimurium* as a function of the relative fluorescence of NPN and increased concentration. Because the exact molecular weight of LPS are difficult to establish for many heterogenous bacterial serotypes, we compared these serotypes on a weight per volume scale. The CAC were 11, 22 and 14 μg LPS/ml respectively. For other several LPS serotypes the CAC are directly proportional to the polysaccharide chain length, if known (Table 1).

Characterization of detergent micelles and incorporated probes. We have earlier established the effectiveness of NPN as a fluorescent marker for detergent micellization in SBP (14). We used the same detergent as a control for the NPN fluorescence assay of bacterial LPS serotypes in this study. The CMC of SBP was estimated to 15 μM in agreement with earlier result (16).

We find that: Fluorescence of free NPN is minimal in this system, as the relative fluorescence is low in the absence of LPS; the location of the NPN molecule in the LPS aggregate is similar to the location of NPN in a SBP detergent micelle e.g. hydrophobic core; CAC for lipid A *S.minnesota* Re 595 and short polysaccharide chain LPS *S.minnesota* Re 595 are very low compared to wildtype LPS; CAC for heterogeneous LPS serotypes with intermediate to long polysaccharide chains (i.e. *S.minnesota* wt; *S.abortus equi, E.coli* 026:B6, *E.coli* 0111:B4, *Se.marcescens and S.typhimurium*) have CAC in the range of 11-25 μg LPS/ml; CAC for heavy MW LPS and mutant strain LPS *E.coli* 0128:B5 *E.coli* 055:B5 were 32 and 38 μg LPS/ml respectively.

DISCUSSION

Characterization of bacterial LPS serotypes. LPS extracted from wild type gram-negative bacteria are heterogeneous in nature making it difficult to establish

TABLE 1

Critical Aggregate Concentration of LPS Serotypes

LPS serotype	MW	[LPS] μg/ml (*μM)
E.coli 0128:B5	ND	32
E.coli 0111:B4	10,000	22
E.coli 055:B5	ND	38
E.coli 026:B6	ND	14
S.minnesota wt	~20,000	11
S.minnesota Re 595	2,500	4 μM*
Lipid A *S.minnesota* Re 595	2,000	5 μM*
S.abortus equi	~5,000–10,000[1]	10
S.typhimurium	~7,000–80,000[2]	14
Se.marcescens	ND	25

Note. CAC estimated from the relative NPN fluorescence intensity at 425 nm, at 20°C, in 0.02 M TRIS, 0.15 M NaCl, pH 7.5. MW's for mutant and wild type LPS were not determined in this study. ND; not yet determined.

[1] MW from [17].

[2] MW from [21].

its molecular weight. In contrast, most short polysaccharide chain mutants yield single molecular weight bands when analyzed with gel electrophoresis (17–19).

It is difficult to interpret the exact mechanisms of LPS macromolecular assembly precisely by fluorescence spectroscopy. Furthermore, the physical organization of LPS polysaccharide chains within and surrounding the LPS aggregates is not well understood and as a result, the exact position of the NPN fluorophores within the LPS aggregates is uncertain.

From the bacterial serotypes of LPS chosen; the short chain mutant *S. minnesota* Re 595, the long chain wild type *S.minnesota, S.abortus equi* and *Se.marcescens, S.typhimurium* and several heterogeneous intermediate chain length *E.coli* 0128:B12, *E.coli* 0111:B4, *E.coli* 055:B5, *E.coli* 026:B6*)* only the short-chain, LPS and lipid A aggregates from *S. minnesota* Re 595 had very low CAC, whereas longer chain LPS have higher CAC. The CAC for heterogenous and wildtype LPS serotypes was determined on a LPS weight/volume for comparison of CAC between different serotypes. Nevertheless, the CAC estimates for the lipid A and the LPS *S.minnesota* Re 595 are comparable to the CMC established for the detergent control.

The observation of intermediate CAC for intermediate chains LPS is consistent with earlier work (14) that the LPS polysaccharide chain extends considerable distances from the lipid A and KDO unit into a third dimension.

The CAC of LPS aggregates reported here are higher than CMC reported by (20) for some purified acyl chain phospholipids. Our higher CAC estimates for LPS aggregates is explained by differences between individual monomer molecular organization, large hydrophilic polysaccharide chain acting as a polar head group, and the more complicated, larger, non-polar acyl chain region present in all LPS molecules.

CAC analysis of LPS serotypes. The heterogeneity in NPN locations inside the LPS macromolecular structure is unquestionably an important issue. The LPS amphiphilic aggregate is unique in several aspects. The diameter of the putative LPS aggregate is nano scale and the exact geometry is a function of both size and solubility of the polysaccharide chains. The wavelength of the emission intensity maximum of NPN shifts from 475 nm to 425 nm as the NPN is incorporated into the LPS aggregates. The Stoke's shift for the NPN molecule also decreased from 125 nm to 75 nm. A change in the Stoke's shift may cause secondary inner filter effects, reabsorption of fluorescence photons and ultimately affect the fluorescence emission and the NPN spectrum. In contrast, NPN fluorescence emission may be affected (by quenching) if NPN molecules are preferentially located directly on or close to the LPS aggregates and within the distance for spectral overlap (R0) for the NPN absorption and emission spectra. However, our experiments were performed with only one concentration of NPN (5 μM) and this did not seem to interfere with increasing fluorescence as the LPS concentration increased.

While little definitive structural data is at present available, for the LPS macromolecular geometry in dilute suspension, data from (14) suggest that the macromolecular interactions of shorter chain LPS serotypes involve geometrical structures in which the polysaccharide chains are in two dimensions. In the longer polysaccharide chain LPS serotypes, the chain extends from the LPS core structure to a third space dimension.

CONCLUSION

The results presented here suggest that LPS, when dispersed into a dilute salt suspension aggregate into micelles, lipidic particles or other macromolecular assemblies at very low molar concentrations. The molecular aggregation is a result of the amphiphilic nature of the LPS monomer which have profound effects on the sar assembly of larger LPS aggregates. The NPN fluorescence method is an effective method for the evaluation of aggregation of amphiphilic macromolecular systems. Additional structural information of macromolecular assembly of LPS in solution is needed to attest structure-function relationship with toxicity of LPS species and to better understand whether LPS interact as single LPS monomers or as macromolecular fragments with specific components in the septic shock pathway.

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