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The supramolecular structure of LPS–chitosan complexes of varied composition in relation to their biological activity

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ABSTRACT

The complexes of chitosan (Ch) with lipopolysaccharides (LPSs) from *Escherichia coli* O55:B5 (*E*-LPS) and *Yersinia pseudotuberculosis* 1B 598 (*Y*-LPS) of various weight compositions were investigated using quasi-elastic light scattering, ζ-potential distribution assay and atomic force microscopy. The alteration of ζ-potential of *E*-LPS–Ch complexes from negative to positive values depending on Ch content was detected. The *Y*-LPS–Ch complexes had similar positive ζ-potentials regardless of Ch content. The transformation of the supramolecular structure of *E*-LPS after binding with to Ch was revealed. Screening of *E*-LPS and *Y*-LPS particles by Ch in the complexes with high polycation was detected. The ability of LPS–Ch complex to induce biosynthesis of TNF-α and reactive oxygen species in stimulated human mononuclear cells was studied. A significant decrease in activity complexes compared to that of the initial LPS was observed only for *E*-LPS–Ch complexes.

1. Introduction

Lipopolysaccharide (LPS or endotoxin) is one of the strongest elicitors of the immune system of macroorganisms. It is responsible for induction of many cytokines and chemokines but also the other immune mediators in human immune cells, such as monocytes, macrophages, dendritic cells, and others (Ulevitch, 2000). Activation of these cells may be beneficial at low amounts of LPS but at higher LPS concentrations lead to pathophysiological reactions such as sepsis (Bone, 1996).

LPS consists of the hydrophobic part, known as lipid A, the repeating O-antigen polysaccharide, and the core oligosaccharide (Raetz, 1990). Many of the immune activation abilities of LPS can be attributed to the lipid A unit, which binds to the toll-like receptor 4 (TLR-4) and activates the host defense effector system by rapidly triggering proinflammatory processes (Peri, Piazza, Calabrese, Damore, & Cighetti, 2010).

Therapies for Gram-negative sepsis remain unsatisfactory despite the concerted effort to develop new treatments for this life-threatening syndrome. Currently, no drugs are specific for endotoxin-induced clinical syndromes. Hence, compounds with therapeutic potentials as anti-endotoxin agents may include those that either bind LPS at high affinity and neutralize its toxicity or those that competitively interact with LPS receptors on host cells without activating biological responses (receptor antagonists). Both groups of agents, acting through different mechanisms, block endotoxin binding to specific receptors on target cells, thus preventing the synthesis of proinflammatory cytokines (Van Amersfoort, Van Berkel, & Kuiper, 2003).

One of the promising ways to inhibit the harmful inflammatory/septic effects of endotoxin is to bind LPS to certain polycations, which can interact with lipid A bearing the negatively charged phosphate groups and, as a result, block this toxic centre of the endotoxin.

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Chitosan, the cationic (1-4)-2-amino-2-deoxy-β-D-glucan, with degree of acetylation typically close to 0.20, is prevalently produced from marine chitin. Chitosan and its partially depolymerized derivatives and oligomers have a wide spectrum of biological activities and are particularly useful in the fields of wound healing, oral delivery, and food industry (Aider, 2010; Muzzarelli, 2009, 2010). The systematic studies of chitosan binding to LPS and the biological properties of the chitosan–LPS complexes showed that it interacts specifically with LPS to form stable water-soluble complexes of various stoichiometric compositions and that the formation of LPS–chitosan complexes is accompanied by significant modification of immunological properties of LPS (Solov'eva, Davydova, Krasikova, & Yermak, 2013).

Due to the amphiphilic nature of their molecules and the very low critical micellar concentration (CMC), LPS exist as aggregates in aqueous solutions and their aggregate state has been found to be an important factor of interaction between LPS and Ch (Davydova, Naberezhnykh, Yermak, Gorbach, & Solov'eva, 2006). As shown previously, the LPS–chitosan complexes are 10–20 times less toxic than LPS alone (Yermak et al., 2006). This effect depends on LPS structure, the ratio between the components in the complex and chitosan molecular weight. A substantial reduction of LPS toxicity in LPS–chitosan complexes may be explained by the blocking of the toxophoric centre of endotoxin or the alterations in the molecular charge and/or the structure of LPS aggregates by chitosan (Solov'eva et al., 2013).

The development of modern polymers analysis techniques offers great opportunities for studying the supramolecular organization of macromolecules. The atomic force microscopy can obtain a high resolution image of the biomolecules under near-physiological conditions. It allows us to study complexes of different stoichiometry between endotoxins of different structure and chitosan and to try to find a correlation between their structure and biological activity. For this purpose two different structural types of LPS (commercial *Escherichia coli* LPS O55 (*E*-LPS) and LPS isolated from bacteria *Yersinia pseudotuberculosis* 1B598 (*Y*-LPS)) and Ch with molecular weight of 110 kDa and *N*-acetylation degree of 1% have been chosen to prepare their complexes with different stoichiometry.

2. Materials and methods

2.1. Isolation of lipopolysaccharides and chitosan

Cells of *Y. pseudotuberculosis* (serovar 1B, strain 598) isolated from a patient suffering from the far-eastern scarlatina-like fever (Institute of Epidemiology and Microbiology, Vladivostok, Russia) were grown at 4 °C in a previously described nutrient medium (Ovodov et al., 1971). LPS from bacterial cells was isolated using the phenol-chloroform-petroleum ether procedure (Galanos, Luderitz, & Westphal, 1969). Nucleic acids were removed by ultracentrifugation at 105,000 × *g*. The purified LPS (yield: 1.2%) contained less than 1% of protein. Protein content was determined by Lowry's method (Lowry, Rosebrough, Farr, & Randall, 1951), nucleic acid contents were estimated in accordance with Spirin et al. (1958). Structural characterization of *Yersisnia pseudotuberculosis* LPS elucidated previously (Krasikova, Gorbach, Solov'eva, & Ovodov, 1978; Tomshich, Gorshkova, Elkin, & Ovodov Yu, 1976; Tomshich, Gorshkova, & Ovodov, 1985).

A preparation of LPS from *E. coli* 055:B5 was purchased from Sigma (Sigma Chemicals, St. Louis, MO, USA).

A chitosan (Ch) sample with molecular weight of 110 kDa and 1% degree of N-acetylation was obtained by alkaline treatment of crab chitin according to the published protocol (Wolfrom & Shen Han, 1959). The degree of *N*-acetylation of the chitosan sample was calculated according to IR-spectroscopy data (Domszy & Roberts, 1985). The molecular weight of the chitosan was determined by viscosimetry in 0.1 M AcOH/0.2 M NaCl according to (Harding, 1997).

2.2. Preparation of LPS–Ch complexes

LPS (1 mg) and Ch (7–1.4 mg) (according to its contents in the complexes) were dissolved separately in 5 ml of pyrogen-free deionized water. The solutions were stored for 48 h at 37 °C; equal aliquots of LPS and Ch were then mixed and incubated for 18 h at 37 °C. The LPS and chitosan solutions were decontaminated of bacteria by filtration using a "Millex GS" filter unit (Millipore, Ireland).

2.3. Dynamic light scattering (DLS) and electrophoretic properties of the LPS–chitosan complexes

The sizes and ζ-potentials of the LPS aggregates and LPS–Ch complexes in solution were determined using a ZetaSizer Nano ZS system (Malvern, UK) operating at 633 nm. Prior to measurements, the samples were left for 1 h to allow the large aggregates to settle, as they can interfere with the measurements even if their content does not exceed a few percent. The measurements were performed at 25 °C for *E*-LPS and at 37 °C for *Y*-LPS. The hydrodynamic diameters of the particles were automatically calculated with the instrument's software based on analysis of the autocorrelation function. The ζ-potentials were calculated from the experimentally determined electrophoretic mobility using the Henry equation (Henry, 1931).

2.4. Atomic force microscopy (AFM)

LPSs were dissolved in distilled de-ionized water at concentration of 0.1 mg/ml; Ch samples—at concentrations of 0.01, 0.1 or 0.5 mg/ml. The LPS–Ch complexes were prepared at the same concentrations. Aliquots (12 μl) of the aqueous solutions of each complex and their initial component were deposited onto freshly cleaved mica and dried at 37 °C for 24 h or at 70 °C for 30 s (for LPS). The morphology of Ch, LPS and their complexes was studied in air by AFM (Solver P47) in the tapping contact mode using a tip with the radius of 10 nm. The topographic parameters of the macromolecular structure have been automatically calculated using the self-developed Balagan's Grain Analysis programme.

2.5. Ethical approval

The study protocol was approved by the medical ethics committee of the local hospital (Vladivostok, Russian Federation). Informed consent was obtained from all subjects who participated in the study. All donors were free of medicines administration for 14 days prior to blood sampling. Blood was drawn from the antecubital vein of normal healthy human volunteers and anticoagulated in plastic tubes (Greiner Bio-One International AG, Kremsmuenster, Austria) with 30 IU lithium heparinate used as an anticoagulant.

2.6. TNF-inducing activity

Human peripheral blood was collected by venipuncture into sterile siliconized tubes and diluted at a ratio of 1:5 with sterile Medium 199 (Sigma, USA) containing 300 mg/l of glutamine (Gibco, Life Technology, Germany) and 50 μg/ml of gentamicin. The diluted blood (0.1 ml) was transferred into sterile polypropylene plates and then incubated with the corresponding LPS, Ch, or LPS–Ch complex (37 °C, 5% CO_2). Control incubation with 1 μg/ml of LPS from *E. coli* (strain 055:B5) was performed for each experiment. After 24 h the supernatants were collected and frozen, followed