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The properties of chitosan complexes with smooth and rough forms of lipopolysaccharides on CHO-K1 cells

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ABSTRACT

The negative charge of LPS molecule and the presence of fatty acids in lipid A structure make it capable of binding with chitosan. In the presented work we analyzed the interactions of chitosan with LPS of *Burkholderia cepacia* or *Proteus mirabilis* and biological effects of these complexes on CHO-K1 cells. We observed that the presence of O-polysaccharide part of LPS (S1959), core region (R110) or lack of fatty acids in lipid A increased binding affinity of endotoxin with chitosan. However, lipid A of *B. cepacia* or *P. mirabilis* R45 might interact with CHO-K1 cells membrane alone or mediated by chitosan, respectively. In conclusion, the presence of two (*B. cepacia*) or one (*P. mirabilis* R45) Ara4N residues in lipid A part, promoted binding to cell membrane of CHO-K1 cells, alone or in the presence of chitosan, respectively. Chitosan reduced biological potencies of *P. mirabilis* lipid A R45 structure and this effect depended on the presence of O-PS. Lipid A of *B. cepacia* induced oxidative DNA damage in CHO-K1 cells.

1. Introduction

The lipopolysaccharide (LPS) called also endotoxin, is a component of the outer membrane of Gram-negative bacteria. The LPS is released from the cell surface of bacteria either in small amounts through its growth and cell division or in large quantities during cell disruption (Caroff & Karibian, 2003). The presence of LPS molecules in the blood stream results in inflammation response of the host (Salomao et al., 2012). Moreover, *in vitro* exposure of endothelial cells to LPS resulted in the reactive oxygen species (ROS) occurrence and cytotoxicity (Choi, Hoffman, Rodway, & Sethi, 2006). These proinflammatory and cytotoxic properties of LPS are linked to its chemical structure. The toxic center of LPS is lipid A, its glycolipid fragment is the most conservative region in LPS structure. In case of *Proteus mirabilis* lipid A R45 structure, the phosphate group at the non-reducing end of GlcN disaccharide is chemically conjugated to 4-amino-arabinose (Ara4N) (Sidorczyk, Zähringer, & Rietschel, 1983). Lipid A of *P. mirabilis* S1959 as well as R110 has two phosphate-linked Ara4N residues (Kondakova, Vinogradov, Lindner, Knirel, & Amano, 2003). *Burkholderia cepacia* lipid A is characterized by two residues of Ara4N linked to reducing and non-reducing phosphate residues (Silipo et al., 2005). The negative charge and amphiphilic nature of the LPS molecule make it capable of binding with ligands whose molecules are positively charged and/or are amphiphilic. Therefore, therapeutic strategies for the treatment of septic shock in humans should be targeted exclusively against the most conserved as well as toxic part of the endotoxin structure, which is lipid A (Jerala & Porro, 2004; Matera et al., 2005, 2012; Mingeot-Leclercq, Tulkens, Denamur, Vaara, & Vaara, 2012).

Previously studies indicated that natural polysaccharides, in particular chitosan, meet the above requirement by virtue of their safety (Yermak et al., 2006; Davydova et al., 2008; Arabski et al., 2009; Muzzarelli, 2010). Chitosan manufactured from chitin is the liner and partly acetylated (1-4)-2-amino-2-deoxy-beta-D-glucan (Muzzarelli et al., 2012). The forming of chitosan-LPS complexes is associated with ionic interactions between negatively charged

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groups on LPS and amino groups on chitosan (Yermak et al., 2006). In practice, chitosan was used for removing endotoxins from biological liquids (Henrie, Ford, Stroup, & Ho, 2011). The major goal of the presented paper was to examine the formation mechanism of complex composed of chitosan and rough (R) or smooth (S) LPS molecules originated from *P. mirabilis*. The role of positively charged substituents Ara4N in *P. mirabilis* lipid A was evaluated as a suspected major domain for interactions with chitosan amino groups. The second element of study was to determine the detoxification efficacy and biological activity of the above complexes tested by genotoxicity, cytotoxicity and immunostimulation assays. Lipid A with two residues of Ara4N originated from *B. cepacia* LPS was used to evaluate the mechanism (proposed in this study) of LPS interactions with CHO-K1 cells.

2. Materials and methods

2.1. Isolation of lipopolysaccharides and chitosan

P. mirabilis O3 (OXK, S1959), and its two R mutants: Ra (R110) and Re (R45) originated from the collection of the Institute of Microbiology and Immunology University of Lodz, Poland. LPS were isolated from bacterial cells by the hot phenol–water method (Westphal and Jann, 1965). The LPS were purified by DNAse and RNAse (Sigma Chemical Co., St. Louis, MO, USA) treatment and ultracentrifugation, as described by Knirel et al. (1997). LPS was essentially free from nucleic acids (about 10.8 μg in LPS solution at 1 mg/ml) and contained less than 2%, w/v of proteins. *P. vulgaris* O3 LPS and its two R mutants were chemically modified by mild basic O-deacylation. LPSOH form is lacking five ester bound fatty acids on its lipid A part. Preparation of lipid A from *B. cepacia* was presented elsewhere (Silipo et al., 2005). A high molecular weight of chitosan sample of 130 kDa and a 4% degree of its N-acetylation was obtained by alkaline treatment of crab chitin according to a protocol published previously (Wolfrom & Shen-Han, 1959). The degree of N-acetylation was calculated according to IR-spectroscopic data (Domszy & Roberts, 1985). The molecular weight of the chitosan was determined by the Archibald method at 12 000 rpm (Archibald, 1947).

2.2. Cell culture

Cytotoxic properties of LPS, chitosan and LPS/chitosan complexes were measured by comet assay using Chinese hamster ovary (CHO-K1) cells. CHO-K1 cells were cultured at 37 °C in a humidified 5% CO_2 atmosphere in plastic dishes in McCoy's 5A medium supplemented with 10% heat inactivated fetal calf serum, 2 mM L-glutamine and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin). We used CHO-K1 as a model of eukaryotic cells for analysis of LPS–chitosan complexes interactions with membrane, no mediated by glycosylphosphatidylinositol-linked glycoprotein – CD14 (Chakravortty et al., 2001).

2.3. LPS/chitosan precipitation assay

Stock solutions of *P. mirabilis* LPS were diluted in 100 μl water at concentrations of 0.03-1 mg/ml prior to the addition of 100 μl colistin at concentration of 1 mg/ml. The reaction mixtures contained LPS, colistin and LPS/colistin complexes in final volumes of 200 μl. Controls were corresponding LPS or colistin solutions. Precipitation proceeded for 18 h at 25 °C and was measured with a time interval $\Delta t = 1$ h. Colistin was used as positive control. Colistin (polymixin E) is a polycationic antibiotic member of the polymixin family. The essential role of hydrophobic and ionic interaction between LPS and polymyxins has been reported (Mares, Kumaran, Gobbo, & Zerbe, 2009). This drug shows amphipathic character and might interact with by fatty acids as well as with carbohydrate moieties of GlcN or Kdo in LPS by polar residues in the cationic decapeptide of polymyxin. On the base of colistin precipitation, the same LPS and chitosan concentration were chosen. Moreover analyzed kinetics of LPS/chitosan complexes formation was performed in the same conditions as colistin. Additionally, O-deacetylated forms of all S and R forms of *P. mirabilis* LPS were applied and the following criteria were applied. Positive result of precipitation ($A_{600\ nm}$) was defined when differences after time 18 h (T_{18}) and time 0 h (T_0) for LPSOH–chitosan formation was higher than total absorbance ($A_{600\ nm}$) of differences between T_{18} and T_0 for LPSOH and chitosan formation. Aggregates were detected by spectroscopic measurement at 600 nm using Microplate Reader TECAN Infinite 200 PRO (Tecan Group Ltd., Switzerland).

2.4. Titration of the Orange II–chitosan complex with LPS solution

To 80 μl of 5 mM phosphate buffer (pH 5.0), 30 μl of Orange II (4-(2-hydroxy-1-naphthylazo) benzenesulfonic acid sodium salt) solution (0.08 mg/ml) and 20 μl of chitosan solution (100 μg/ml) were added. The mixture was incubated for 20 min at 25 °C. Then the resulting complex was titrated with LPS solution (200–150 μg/ml), preincubated at 37 °C for 48 h, by adding different portions of LPS. The mixtures were incubated at 37 °C until a constant mean of optical density of the solutions during 24 h (LPS O3 and R110) or 48 h (LPS R45) was obtained. The absorption was determined with a μQuant spectrophotometer (Bio-TEK Instruments, USA) at 483 nm in three parallel samples, and the mean arithmetic value was calculated. The value of $\Delta D = D_{exp} - D_0$ was calculated, where D_0 and D_{exp} were absorptions of the solutions before and after the addition of LPS, respectively. The values of ΔD_{max} and K_b were determined from the Scatchard plot in $\Delta D/C_{LPS}$ versus ΔD coordinates. The chitosan saturation (Q) with LPS molecules was calculated from the ratio $\Delta D/\Delta D_{max}$. The degree of cooperativity (h) was determined from the Hill equation in logarithmic form (Hill, 1910):

$$\log \frac{Q}{1-Q} = h \log[C_{LPS}] - \log[K_b]$$

The number of binding sites on the endotoxin molecule per glucosamine unit of chitosan was assessed from the saturation curve by plotting a tangent to the point that corresponded to the maximal change in the reaction mixture's absorption. Then, the LPS concentration corresponding to the chitosan saturation with the endotoxin was determined.

2.5. Limulus amebocyte lysate (PyroGene rFC) assay

The endotoxin level in chitosan stock solutions and O3, R110 or R45 LPS *P. mirabilis* inactivated by chitosan was determined by the fluorogenic endotoxin detection assay PyroGene rFC purchased from Lonza (Walkersville, USA) in accordance with the test instruction. The fluorescence was measured with Microplate Reader TECAN Infinite 200 PRO (Tecan Group Ltd., Switzerland).

2.6. Complement activation

To assess complement system activation by the chitosan polymers in vitro, the quantitation of terminal complement complex SC5b-9 in human serum was analyzed using enzyme-linked immunosorbent assay kits from Quidel Corporation (San Diego, USA). The chitosan (50 or 500 μg/ml) in veronal-buffer saline containing 0.15 mM Ca^{2+} and 0.5 mM Mg^{2+}, pH 7.4 were incubated with fresh human serum from healthy volunteers at a volume ratio of 1:9 at 37 °C for 60 min. After incubation with gentle agitation (80 rpm), the serum samples were centrifuged (2500 rpm,

10 min), serially diluted in PBS solution containing 0.05% Tween-20 protein stabilizers and 0.035% ProClin 300, and SC5b-9 concentration was measured according to manufacturer's instructions. Zymosan (Sigma) at final concentration 50–500 μg/ml, a yeast cell wall extract was used as a positive control. Negative control samples consisted of human serum only.

2.7. ELISA

ELISA method was used to analyze the binding of LPS mediated by chitosan to CHO-K1 cells. The plates with CHO-K1 cells monolayer (1×10^5) were preincubated for 30 min with chitosan at 50 μg/ml and treated with LPS at 100 μg/ml for 1 h at 37 °C. The plates were incubated with rabbit polyclonal anti-LPS sera (diluted in PBS 1:1000) for 1 h at 37 °C and washed three times with PBS. Diluted (1:1000) goat anti-rabbit IgG conjugated with peroxidase (Sigma, USA) in 1% BSA in PBS was used as the second antibody. Next, the plates were washed three times with PBS. A solution of 0.4 mg *o*-phenylenediamine dihydrochloride (Sigma, USA) was freshly prepared and added. After 25 min of incubation at ambient temperature, the reaction was stopped by adding 0.5 M H_2SO_4, and the absorbance was measured at 490 nm with Microplate Reader TECAN Infinite 200 PRO (Tecan Group Ltd., Switzerland).

O-antisera against *P. mirabilis* S1959, R110 or R45 were obtained by immunization of rabbits with heat-inactivated bacteria (100 °C, 2.5 h). To prepare the immunogen, lyophilized bacteria were suspended in sodium chlorine (0.85% NaCl) at a concentration of 1 mg/ml. Animals were injected intravenously with 50, 100, 100, 200 and 500 μg of immunogen in 500 μl of saline solution on days 1, 4, 7, 11 and 16, respectively. Seven days after the last immunization, the blood was sampled by the cardiac puncture, and then serum was separated and stored in 0.5 ml aliquots at −20 °C. The experiments were carried out in accordance with Animal Ethical Committees guidance.

2.8. DNA damage (comet assay)

CHO-K1 cells were preincubated for 30 min with chitosan at 50 μg/ml and treated with LPS at 100 μg/ml for 1 h at 37 °C. DNA damage was assessed by the alkaline comet assay according to Wojewódzka, Kruszewski, Iwanenko, Collins, & Szumiel, 1998. In free radicals scavenging experiments CHO-K1 cells were pretreated with dimethyl sulfoxide (DMSO). The cells were incubated with DMSO at 0.5 mol/l for 30 min at 37 °C.

2.9. Apoptosis

CHO-K1 cells were preincubated for 30 min with chitosan at 50 μg/ml and treated with LPS at 100 μg/ml for 1 h at 37 °C. After the treatment, the frequencies of early apoptotic, late apoptotic and necrotic cells were evaluated with the Annexin V-FITC apoptosis detection Kit I (BD Pharmingen, USA) described elsewhere (Darzynkiewicz, Huang, Okafuji, & King, 2004).

2.10. Data analysis

The data were analyzed using the Statistica (StatSoft, Tulsa, OK, USA) software package. Analysis was made in triplicate in one (ELISA, LAL, titration of the Orange II–chitosan complex with LPS solution) or in two independent experiments (complement activation, DNA damage, apoptosis). All the values in this study are expressed as mean ± SD. If no significant differences between variations were found as assessed by the Snedecor–Fisher test, the differences were compared by the one-way ANOVA test.

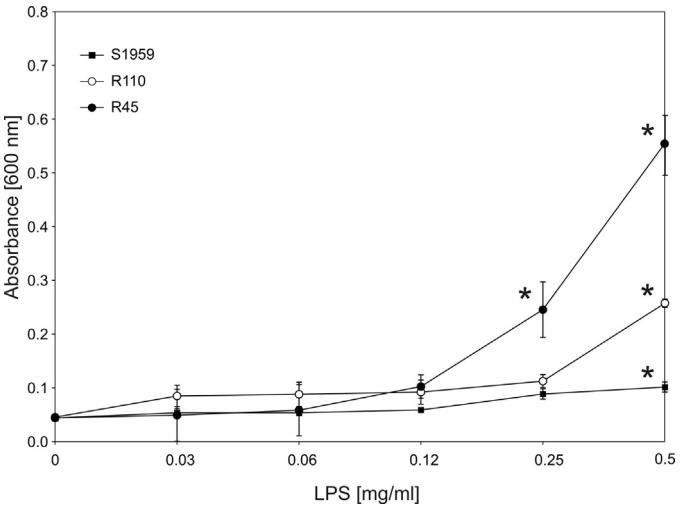


Fig. 1. Precipitation of colistin (0.5 mg/ml) with S1959, R110 and R45 LPS *P. mirabilis* proceeded for 18 h at 25 °C. Colistin/LPS aggregates were detected by spectroscopic measurement at 600 nm. The results are the means of three independent experiments. * $p < 0.05$.

3. Results

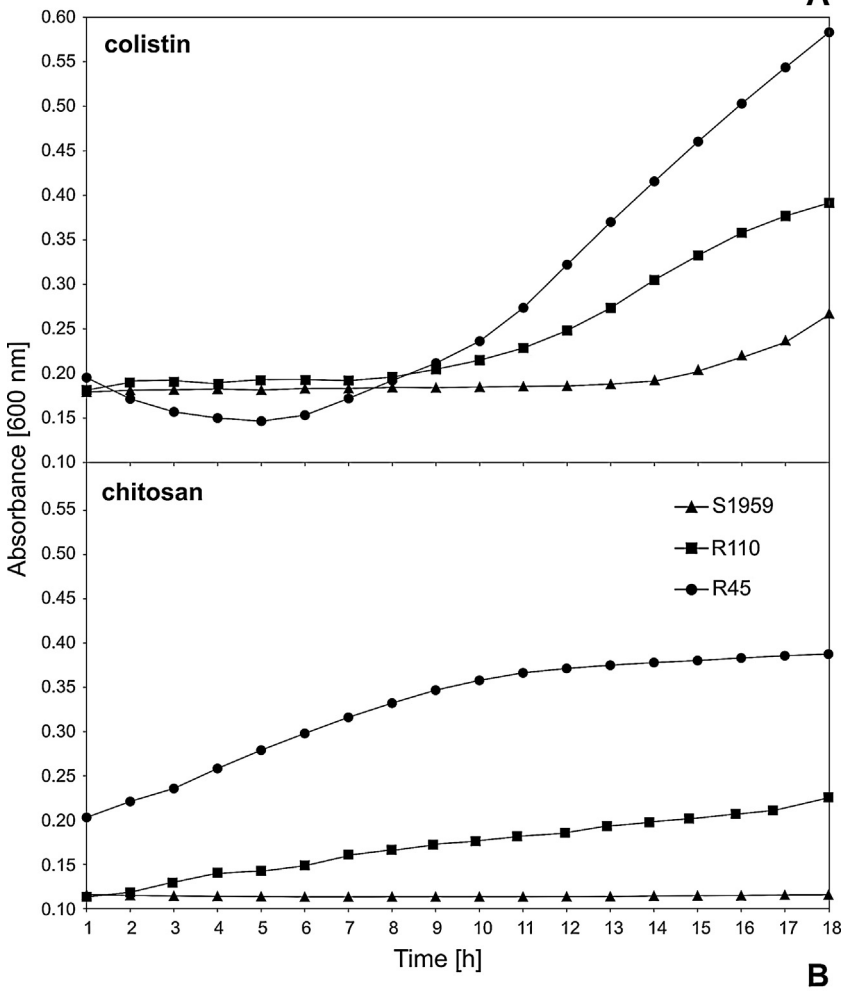
3.1. Aggregates formation of LPS with chitosan

The interaction of endotoxin with chitosan or colistin may result in aggregate formation detected by absorption values at 600 nm. Fig. 1 shows precipitation of colistin (0.5 mg/ml) with a varied amount of S1959, R110 or R45 *P. mirabilis* LPS proceeded for 18 h at 25 °C. Statistically significant amounts of LPS with colistin aggregates were noticed at the concentrations of 0.25 and 0.5 mg/ml for both reagents, respectively. The amount of precipitates depended on the content of lipid A in *P. mirabilis* LPS. Fig. 2A shows different kinetics of S1959, R110 and R45 LPS aggregates formation with chitosan in comparison to LPS/colistin. The most effective precipitation was observed for R45 LPS and colistin. Interestingly, the precipitation during the first 7 h of incubation with colistin was decreased, in contrast to chitosan. This effect might be associated with disruption of R45 micelles by colistin, probably by detergent-like mode of action. In addition, the amount of precipitate complexes of chitosan/R45 was lower than colistin/LPS after 18 h of incubation. To establish the role of lipid A fatty acids in precipitation process with chitosan, O-deacetylated LPS were used. We observed weaker aggregate formation of LPSOH–chitosan complexes (Fig. 2B).

3.2. The determination of binding parameters of LPS–chitosan

The binding constants of the lipopolysaccharides with the chitosan were determined using the displacement of the anionic dye tropaeolin 000 N2 (sodium salt of 4-(2-hydroxy-1-naphthylazo) benzenesulfonic acid) by lipopolysaccharide in its complex with chitosan. The dye has been previously shown (Glazunov & Gorbach, 1999) to bind to every ionized amino group in the polycation molecule, resulting in decreased dye absorption (to 70%) at 483 nm. On addition of LPS solution to the tropaeolin–chitosan complex, the absorption of the reaction mixture at 483 nm increased to the value corresponding to that of the free dye. This indicated that the dye was displaced by lipopolysaccharide from its complex with chitosan and the endotoxin-polycation complex was formed (Davydova, Naberezhnykh, Yermak, Gorbach, & Solovèva, 2006). No effect of LPS on the absorption of tropaeolin was recorded. Data on LPS binding with chitosan, presented as linear Scatchard

A



B

LPS	$T_{18}(A_{600\,nm}) - T_0(A_{600\,nm})$			
	LPS (1)	chitosan (2)	LPS/chitosan (3)	Result; (3)>(2+1)
S1959	0.015	0.002	0.011	-
S1959-OH	0.005	0.002	0.008	-
R110	0.094	0.002	0.180	++
R110-OH	0.004	0.002	0.030	+
R45	0.135	0.002	0.175	+++
R45-OH	0.001	0.002	0.019	+

C

LPS	$K_b \times 10^6$, M (Scatchard)	$K_b \times 10^5$, M (Hill)	$K_b \times 10^5$, M (mean value)	h*	Number of LPS moles per glucosamine unit of chitosan
S1959	1.690	1.760	1.725	0.998	0.089
R110	0.127	0.126	0.126	0.991	0.328
R45	0.072	0.077	0.075	1.015	0.544

Fig. 2. (A) The precipitation kinetics of colistin (upper panel) and chitosan (lower panel) at 0.5 mg/ml concentration with S1959, R110 and R45 *P. mirabilis* LPS measured for 18 h at 25 °C. (B) Precipitation of O-deacetylated (LPS-OH) and native forms of P. mirabilis LPS incubated with chitosan for 18 h at 37 °C. Results are presented as absorbance differences between T_{18} and T_0. (C) Parameters of the LPS binding by chitosan determined by titration of the Orange II–chitosan complex with LPS solution. h^* – Hill's coefficiency.

plots, suggest that the interaction occurs at independent sites of the same type and that cooperativity during the interaction is absent (Fig. 2A). The values of the cooperativity coefficients determined from the Hill plot (Hill, 1910) are close to unity, confirming our suggestion (Fig. 2C). The binding constants for the chitosan-LPS complexes were determined from the Scatchard and Hill plots assuming the independence of the binding sites on chitosan. The LPS concentration corresponding to the point of chitosan saturation by endotoxin was determined from the saturation curves and the number of binding sites was calculated.

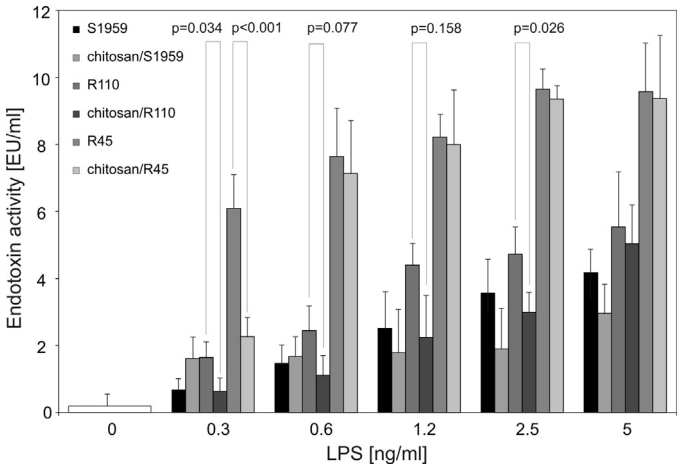


Fig. 3. The level *P. mirabilis* LPS detected in mixtures with chitosan at 100 μg/ml after incubation for 18 h at 37 °C measured by PyroGene rFC assay.

3.3. PyroGene rFC assay

The PyroGene test utilizes recombinant Factor C (rFC) which is used in combination with a fluorogenic substrate. The rFC recognizes only biologically active endotoxin (lipid A) without glucans. We observed the relatively low level of environmental endotoxin detected in chitosan isolates: 0.68 EU/ml in doses of 100 μg/ml. Fig. 3 shown that the amount of detected LPS by the PyroGene test increased with doses used, the most active being R45 LPS. In low LPS doses (0.3 ng/ml) chitosan neutralized toxicity of LPS R45 and R110 ($p < 0.001$ and $p = 0.034$, respectively). Chitosan reduced LPS activities from 6.09 EU/ml to 2.26 for R45 LPS at concentration 0.3 ng/ml. Chitosan decreased the level of R110 LPS at concentration 2.5 ng/ml from 4.73 EU/ml to 2.99 EU/ml ($p = 0.026$). S1959 LPS at doses of 1.2, 2.5 or 5 ng/ml LPS has lower, but not statistically significant, activity in the presence of chitosan.

3.4. Human complement activation by chitosan

Carbohydrate polymers (i.e. chitosan) may activate human complement via classical, alternative or lectin pathways. All three pathways end up on the membrane attack formation (MAC) detected by the presence of C5b-C9. The activation of the human serum complement system by chitosan is shown in Fig. 4. The chitosan used at final concentration of 500 μg/ml activated serum complement to an extent that was 2.3-fold lower as compared with activation caused by 500 μg/ml of zymosan, a well known complement system activator. The ten times lower concentration of chitosan (50 μg/ml) activated complement approximately 40-times stronger compared to human serum alone (negative control) and 4.5-fold weaker than 500 μg/ml of zymosan (positive control).

3.5. ELISA

To find out if *P. mirabilis* S and R LPS binding to CHO-K1 cells is modified by chitosan, the amount of LPS was monitored by rabbit antibodies, specific for each LPS. Fig. 5 shows the levels of specific anti-S or -R form rabbit antibodies recognizing *P. mirabilis* LPS adhered on CHO-K1 cells surface. We observed that the level of anti-R LPS form antibodies was higher for CHO-K1 cells preincubated for 0.5 h with chitosan at 50 μg/ml in contrast to native R45 and R110 at concentration 100 μg/ml (0.002 and 0.009, respectively). We observed statistically significant ($p = 0.001$) binding the

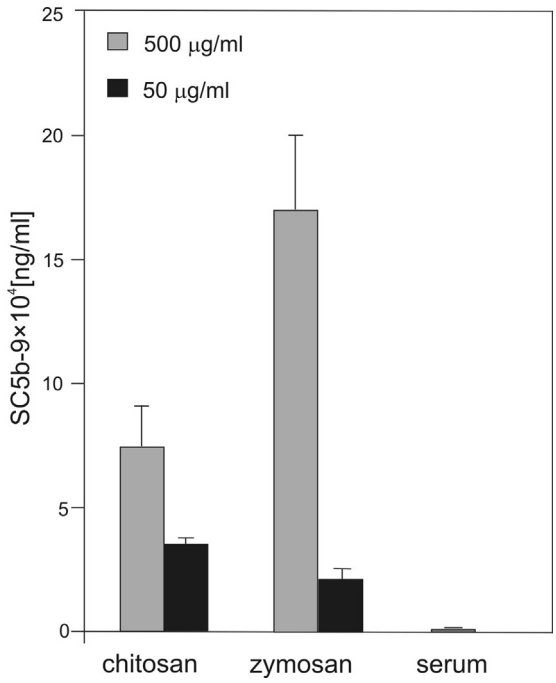


Fig. 4. Human serum complement system activation by chitosan. A representative experiment performed at least in duplicate is presented.

anti-S1959 LPS antibodies to CHO-K1 cells after preincubation with chitosan.

3.6. DNA damage

To determine the role of charged components of LPS the *B. cepacia* Re type LPS as internal control was used. *B. cepacia* LPS differs from *P. mirabilis* Re (R45) by an additional residue of 4-amino-arabinose at the non-reducing end of lipid A molecules. The DNA damage induced in CHO-K1 cells by chitosan/LPS alone or in complexes was determined by alkaline single-cell gel electrophoresis (comet assay) (Fig. 6A). Chitosan or three forms of *P. mirabilis* LPS alone did not induce DNA damage in CHO-K1 cells. The highest level of single- and double-strand DNA breaks in CHO-K1 cells was detected after 1 h incubation at 37 °C with Re form of *B. cepacia* LPS ($p = 0.008$). Chitosan reduced the DNA damage level significantly ($p = 0.061$). Additionally, DMSO (as free radical scavenger) decreased the extent of DNA damage evoked by *B. cepacia* LPS ($p = 0.076$) (Fig. 6B). The contrary results were obtained for R45 LPS, where chitosan increased DNA damage ($p = 0.067$).

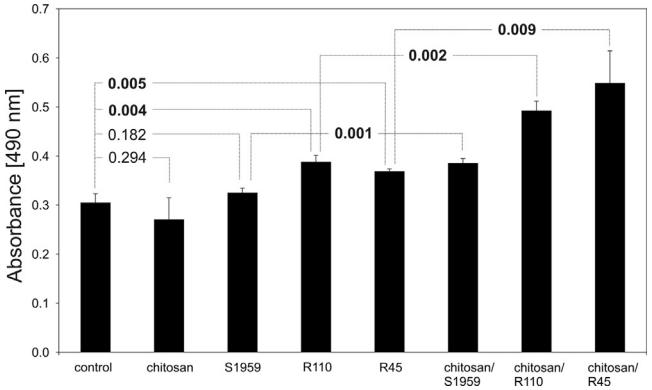


Fig. 5. Detection of LPS on the CHO-K1 cells surface preincubated with chitosan at 50 μg/ml measured by ELISA.

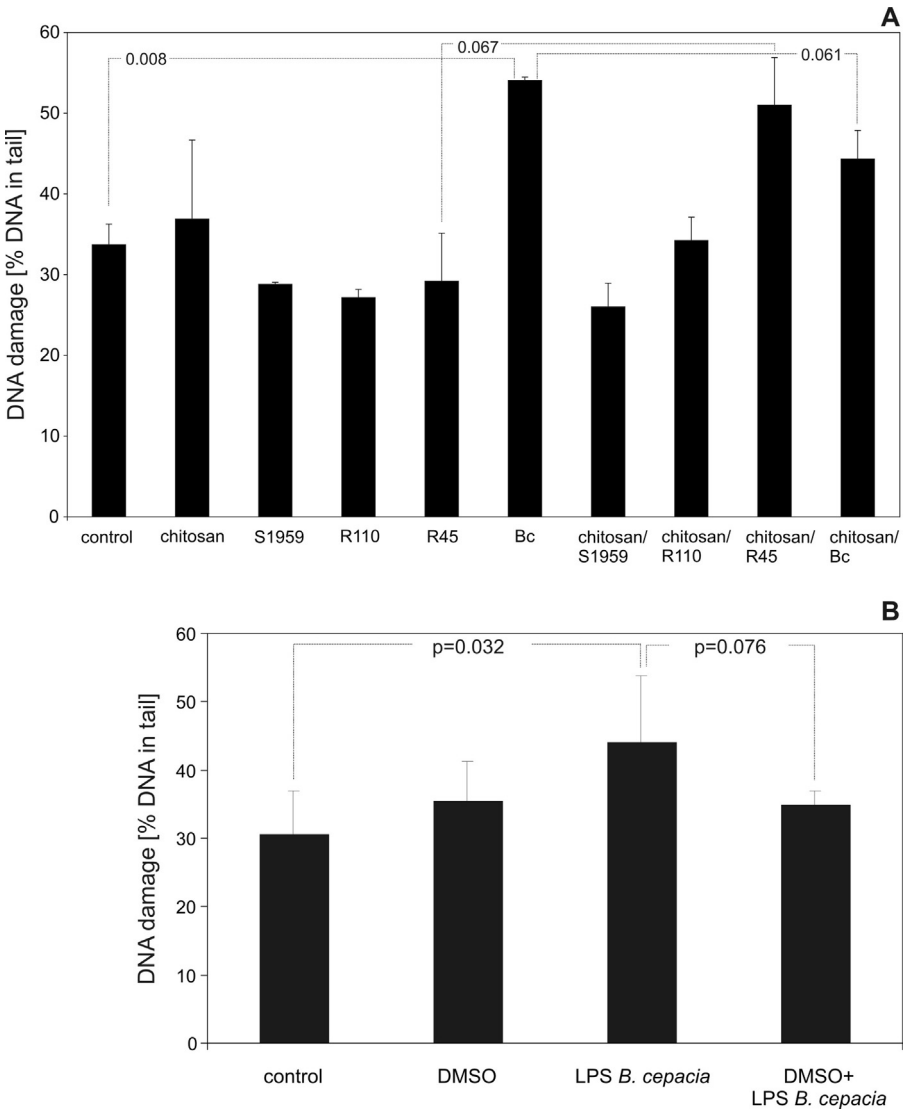


Fig. 6. (A) DNA damage in CHO-K1 cells caused by chitosan at 50 μg/ml and LPS at 100 μg/ml and (B) by DMSO at 50 μg/ml and LPS from *B. cepacia* at 100 μg/ml analyzed by comet assay. Bc–LPS of *B. cepacia*.

3.7. Apoptosis

The amount of Annexin and IP allowed to determine the apoptosis and necrosis of CHO-K1 cells in the presence of LPS and chitosan alone or in complexes (Table 1). We analyzed proapoptotic properties of both reagents in the same condition as applied in studies using comet assay. A number of early and late apoptotic cells was detected after incubation with LPS at 100 μg/ml (1 h) or after preincubation with chitosan at 50 μg/ml (0.5 h) at 37 °C. The flow cytometric analyses of the CHO-K1 cells population showed that the distributions of early and late apoptotic cells (%) in the presence of LPS alone or in complexes with chitosan were similar to controls (untreated cells or preincubated with chitosan).

4. Discussion

The positively charged chitosan interacts with negatively charged groups on LPS: phosphate substitutes located on lipid A and carboxylic groups in the O-specific polysaccharide (Arabski et al., 2009; Davydova et al., 2008; Yermak et al., 2006). This interaction might be modulated by positively charged substituent in lipid A structure, like Ara4N at the non-reducing end of GlcN disaccharide in *P. mirabilis* R45 LPS structure. Moreover, ester bound fatty acids play an important role in aggregates formation by chitosan with *P. mirabilis* LPS as was shown by precipitation assay (Fig. 2). The presence of polysaccharide part of LPS (inner core oligosaccharide substituted with ethanolamine phosphate in R110 and/or O-polysaccharide in S1959) reduced the amount of precipitates. It indicates that hydrophobic nature of LPS plays a predominant role in LPS–chitosan precipitation process (Fig. 2B and C). The binding constant of LPS with chitosan decreases dramatically with increasing hydrophobic character of the LPS.

Theoretically, the presence of Ara4N in lipid A should attenuate the ionic interactions between lipid A and chitosan. Now it is convention fact that cation binding to LPS can be interpreted, at least initially, to be electrostatic and to result from the attraction between the positive charges of peptides and the negative charges (phosphates and carboxylates) of LPS (Andra et al., 2005)

Really, S-LPS from *Proteus* spp. has $K_b = 1.73 \times 10^{-5}$ (Fig. 2C) that is about four time less than for *E. coli* 055:B5 LPS ($K_b = 7.95 \times 10^{-5}$ M) determinated by us early (Davydova et al., 2008) and in about 1.5 time less than for S-LPS from *Y. pseudotuberculosis* ($K_b = 2.8 \times 10^{-5}$ M) (Davydova et al., 2006).

Table 1
Percentage of early and late apoptotic and necrotic CHO cells preincubated with chitosan (50 μg/ml) and treated with LPS (100 μg/ml); mean of two independent experiments ± SD.

Substance	Normal cells (Annexin−/IP−)	Apoptosis		Necrosis (Annexin−/IP+)
		Early (Annexin+/IP−)	Late (Annexin+/IP+)	
Control	94.60 ± 2.97	2.10 ± 0.71	1.45 ± 0.35	1.85 ± 1.91
Chitosan	91.15 ± 5.44	1.55 ± 0.35	0.95 ± 0.78	6.40 ± 5.80
LPS R45	91.25 ± 6.58	6.15 ± 5.87	0.90 ± 0.85	1.75 ± 1.48
Lipid A Bc	93.20 ± 1.70	3.05 ± 1.34	2.15 ± 0.49	1.50 ± 0.85
Chitosan/LPS R45	83.30 ± 5.52	4.00 ± 1.27	3.00 ± 0.71	9.75 ± 4.88
Chitosan/lipid A Bc	89.35 ± 4.17	3.90 ± 1.84	2.10 ± 0.14	4.60 ± 2.55

IP – propidine iodine.

The amphiphilic character and aggregation properties of LPS determine its supramolecular structure and the phenomenon of micelles formed by LPS is well documented (Seydel, Brandenburg, Koch, & Rietschel, 1989). Lamellar or inverted cubic structures of LPS may occur as mixed states under different physiological conditions and have far-reaching implications for the induction of various biological effects (Seydel et al., 1989). In this study different kinetics of colistin and chitosan interactions with LPS *P. mirabilis* R45 were observed (Fig. 2A) what can detect LPS aggregates disruption in colistin treatment, in contrast to micelle promotion done by chitosan. Similar results of colistin actions were described previously by (Zavascki, Goldani, Li, & Roger, 2007; Li, Nation, Milne, Turnidge, & Coulthard, 2005). Moreover, the positively charged colistin molecules can be neutralized by deacetylated O25 LPS *P. vulgaris* (lactic acid in O-polysaccharide) promoting LPS-colistin complex formation and its diffusion through membranes (Arabski, Wąsik, Dworecki, & Kaca, 2007). These findings are in accordance with the bioinformatic model proposed by Mares et al., 2009.

According to summarized data of toxicities on animals cells of most chitosans (and derivatives) are not significantly toxic (Kean, Roth, & Thanou, 2005). Chitosan might interact with the negatively charged phosphate groups on the CHO-K1 cells membrane and did not induce damage to DNA (Fig. 6). The preincubation with chitosan evoked interactions between S forms of *P. mirabilis* LPS with CHO-K1 cells (Fig. 5). Probably, chitosan mediated the binding of LPS micells (carboxylic groups in the O-specific polysaccharide) with negative charged cell surface of CHO-K1 (Fig. 7A and B). This assumption accords well with our previous data (Davydova et al., 2008), where the interactions of LPS deriving from different microorganisms with chitosan resulted in the formation of

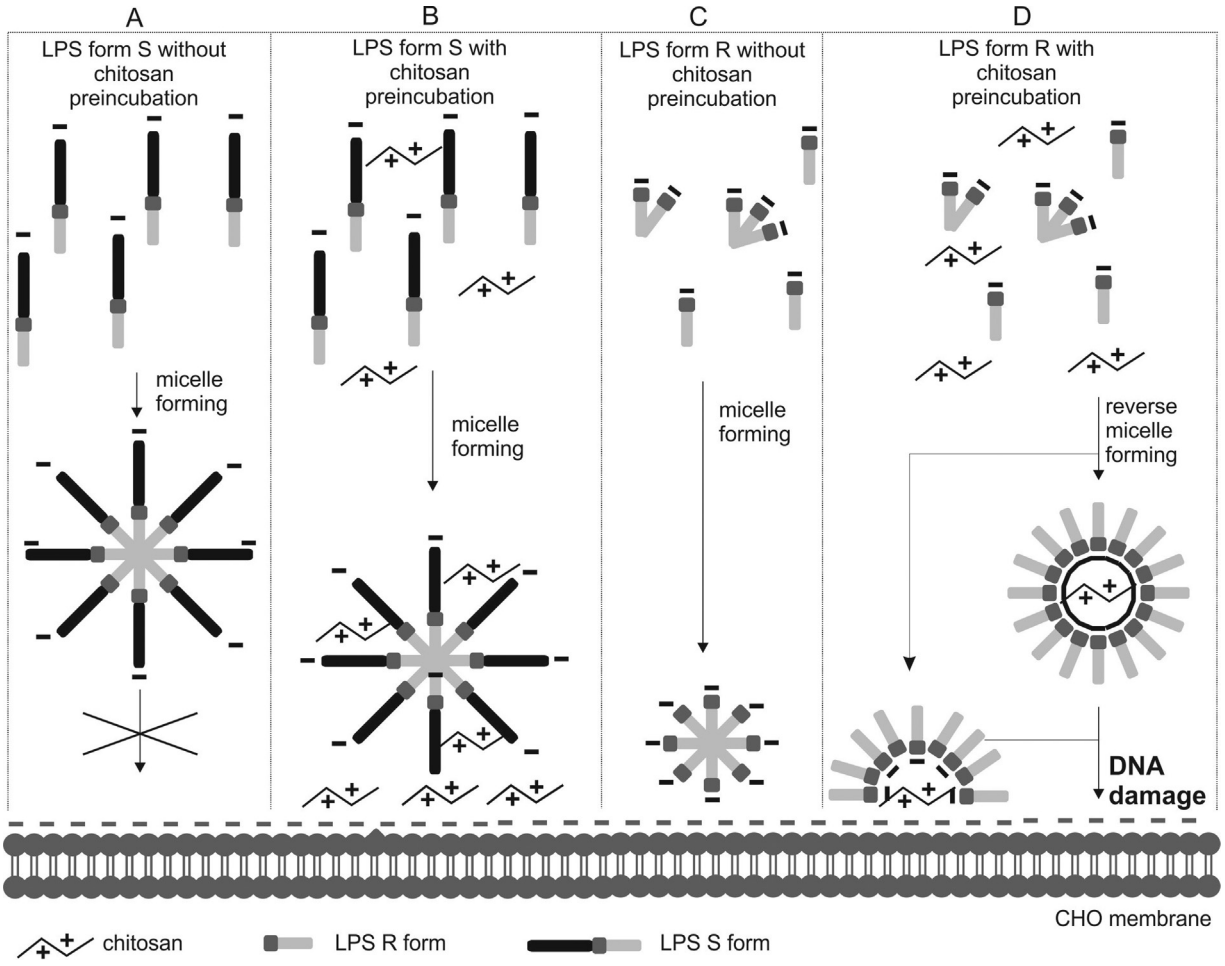


Fig. 7. Probable mechanism of genotoxicity mediated by LPS R forms and chitosan interactions.

complexes in which the negative charges of LPS were neutralized (*E. coli*) or overcompensated (*Y. pseudotuberculosis* and *P. vulgaris*). Additionally, the presence of chitosan facilitated the S and R forms LPS *P. mirabilis* fixation to CHO-K1 cells surface membranes (Fig. 5). The binding of R45 LPS *P. mirabilis* mediated by chitosan promoted strands breaks in DNA of CHO-K1 cells, in contrast to S1959 and R110 LPS binding (Fig. 6). We suggest that Ara4N residues in structure of R45 *P. mirabilis* as well as chitosan molecules might allow ionic interactions with phosphate groups of CHO cell membrane and promote hydrophobic interactions between fatty acids of lipid A from R45 and CHO-K1 membrane (Fig. 7C and D).

The other possible mechanisms of the chitosan complex formation with R-forms of LPS cannot be excluded. According to the chemical structures of the R-LPS it is possible to expect that it will form densely packed aggregates of larger size as S-forms (Brandenburg, Funari, Koch, & Seydel, 1999; Davydova, Ermak, Gorbach, Drozdov, & Solovèva, 2000; Davydova et al., 2008). Interaction of R-LPS with chitosan can probably result in formation of large positively charged particles which can bind to phosphate groups in CHO-K1 cells with higher affinity than neutral S-LPS–chitosan particles.

This R-LPS with chitosan complex formation on cell surface affects an induction of DNA damage measured by comet assay, in contrast to parent R45 (Fig. 7A). The mechanisms of DNA damage inductions by chitosan-R45 *P. mirabilis* complexes are unclear. However, some investigators determined the role of ROS and reactive nitrogen intermediates (RNI) in LPS-induced cytotoxicity of endothelial cells (Sylte, Inzana, & Czuprynski, 2004). Generally, DNA strand breaks might be generated by endonucleases, activated in the late stage of apoptosis process. Analysis of proapoptotic properties of R45–chitosan complexes shown that induction of DNA strand breaks is not associated with activation of endonucleases (Table 1). We suggest that free radicals are involved in oxidative modifications of DNA bases which might be converted into strand breaks by DNA damage repair systems and indirectly detected by comet assay. No antioxidant properties of chitosan against DNA damage induced by R45–chitosan complexion formed in CHO-K1 cells membrane were observed. Probably, N-substituted chitosan by phosphate groups of lipid A (R45) and/or membrane phospholipids has weak antioxidant properties. Lipids A of *P. mirabilis* S1959 and R110, similar as *B. cepacia*, have two phosphate-linked Ara4N residues. The presence of negatively charged residues in O-polysaccharide region decreased interactions of S1959 LPS with CHO-K1 cells membranes in contrast to R110 and R45 LPS. However, chitosan mediated binding of these LPS with cell membrane (Fig. 5). The inner core oligosaccharide of LPS *P. mirabilis* of R110 is substituted with ethanolamine phosphate. Thus, to evaluate the role of Ara4N in LPS interactions with CHO-K1 cells membranes mediated by chitosan, lipid A from *B. cepacia* was used as control. The confusing fact was that the parent *B. cepacia* Re LPS had similar genotoxic properties as *P. mirabilis* Re did in complexion with chitosan (Fig. 6). It is proposed that the interactions between phospholipids of cell membrane and two Ara4N in *B. cepacia* Re LPS are stronger than one Ara4N and phosphate group on non-reducing end of GlcN disaccharide mediated by chitosan molecule. Additionally, chitosan protected CHO-K1 cells against DNA damage induced by *B. cepacia* Re LPS (Fig. 6). This effect might by associated with weaker interactions of chitosan with cell surface than Ara4N and as not fully N-substituted possess antioxidant properties. The experiments with membrane permeable ROS scavenger (DMSO) confirmed the role of free radicals in DNA damage induction by parent *B. cepacia* Re LPS.

We concluded that chitosan might be considered an anti-endotoxin agent but the biological properties of formed complexes with LPS, such as gentotoxicity or complement activation, should be studied in the future.

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