Effects of *Proteus mirabilis* Lipopolysaccharides with Different O-Polysaccharide Structures on the Plasma Membrane of Human Erythrocytes

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The effects of O33 and O49 *P. mirabilis* lipopolysaccharides (LPSs) on human erythrocyte membrane properties were examined. Physical parameters of the plasma membrane, such as membrane lipid fluidity, physical state of membrane proteins, and osmotic fragility, were determined. The fluidity of the lipids was estimated using three spin-labeled stearic acids of doxyl derivatives: 5-doxylstearic acid, 12-doxylstearic acid, and 16-doxylstearic acid. All the applied labels locate to different depths of the lipid layer and provide information on the ordering of phospholipid fatty acyl chain mobility. LPSs O49 increased the membrane lipid fluidity in the polar region of the lipid bilayer as indicated by spin-labeled 5-doxylstearic acid. An increase in fluidity was also observed in the deeper region using 12-doxylstearic acid only for O33 LPSs. The highest concentration of O33 LPSs (1 mg/ml) increased the motion of membrane proteins detected by the spin-label residue of iodoacetamide. These results showed different actions of O33 and O49 LPSs on the plasma membrane due to the different chemical structures of O-polysaccharides. *P. mirabilis* O33 and O49 LPSs did not induce changes in the membrane cytoskeleton, osmotic fragility and lipid peroxidation of erythrocytes. On the other hand a rise in the content of carbonyl compounds was observed for the highest concentrations of O33 LPS. This result indicated protein oxidation in the erythrocyte membrane. Lipid A, the hydrophobic part of LPS, did not change the membrane lipid fluidity and osmotic fragility of erythrocytes. Smooth and rough forms of *P. mirabilis* LPSs were tested for their abilities for complement-mediated immunohemolysis of erythrocytes. Only one out of seven LPSs used was a potent agent of complement-mediated hemolysis. It was rough, Ra-type of *P. mirabilis* R110 LPS. The O-polysaccharide-dependent scheme of reaction is presented.

Key words: Erythrocyte, *Proteus mirabilis* Lipopolysaccharide, Membrane Fluidity

Introduction

Bacteria of the genus *Proteus* of the family Enterobacteriaceae are facultative human pathogens responsible for wound and burn infections as well as skin, eye, ear, nose, throat, urinary tract, and gastrointestinal infections and bacteremias (Morrison and Ryan, 1987). The role of *Proteus* surface antigens in the pathogenesis of rheumatoid arthritis has also been suggested (Dunn, 2000). The outer-membrane lipopolysaccharide (LPS) is considered an important virulence factor of *Proteus*. The polysaccharide chain (O-polysaccharide or O-antigen) and sometimes the core region of the LPS define the serological specificity of these bacteria. Currently, *Proteus* strains are classified into about 80 O-serogroups. In most O-serogroups, the O-polysaccharide is acidic due to the presence of uronic acids, aldulosonic acids, amino acids, phosphate, and other acidic non-sugar components. Two representative LPSs of *Proteus mirabilis*, O33 and O49, that differ in their O-specific polysaccharide (OPS) structures were chosen for studies of interaction with the cell membranes of human erythrocytes.

The complete structure of *P. mirabilis* PrK 59/57 O33 of OPS of LPS was determined (Toukach *et al.*, 2002; Gmeiner *et al.*, 1997). A tetrasaccharide repeating unit of the OPS consists of two residues of D-GlcNAc and one residue each of D-Gal, D-Glc, ethanolamine, and ribitol phosphates, the substitution with Etn*P* being non-stoichiometric (~75%) (for structures see Table I) (Toukach *et al.*, 2002; Gmeiner *et al.*, 1997). The presence of Glc6*P*Etn and/or Rib-ol-5-*P* in the OPS of O33

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Table I. Structures of the O-polysaccharides of *Proteus mirabilis* used in the hemolysis assay.

Strain	Structure of the repeating unit	Reference
P. mirabilis O7 (PrK 15/57)	β-D-Qui*p*4NMal-(1¬ 6 →2)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→3)-β-D-Glc*p*NAc-(1→	Kondakova *et al.* (2004)
P. mirabilis O3a,3b (OXK, S1959)	α-D-Gal*p*A6(L-Lys)-(1¬ 4 α-D-Glc*p*-(1¬ 2 →6)-β-D-Gal*p*NAc-(1→4)-β-D-Glc*p*A-(1→3)-β-D-Gal*p*NAc-(1→	Ziółkowski *et al.* (1997)
P. mirabilis O33 (PrK 59/57)	Rib-ol-5-*P*¬ 3 EtnP¬ 6 →2)-β-*D*-Gal*p*-(1→3)-α-*D*-Glc*p*NAc-(1→3)-β-*D*-Glc*p*-(1→3)-β-*D*-Glc*p*NAc-(1→	Toukach *et al.* (2002)
P. mirabilis O38 (PrK 64/57)	AcEtn*P*¬ 6 →3)-β-D-*p*4N(Ac-D-Asp)-(1→6)-α-D-Glc*p*-(1→3)-α-D-Gal*p*A-(1→4)-α-D-Glc*p*NAc-(1→	Kondakova *et al.* (2003)
P. mirabilis O49 (PrK 75/57)	α-D-Quip4NSuc-(1¬ 4 →2)-α-D-Gal*p*A-(1→3)-α-L-Rha*p*-(1→4)-α-D-Glc*p*-(1→2)-α-L-Rha*p*-(1→ 3)-β-D-Glc*p*NAc-(1→	Kondakova *et al.* (2004)

LPS resulted in a negative charge of the whole LPS molecule.

The second *P. mirabilis* PrK 75/57 (O49) LPS used contains a unusual acyl derivative of Qui4N-4, 6-dideoxy-4-succinamido-D-glucose on its OPS (Table I). In addition to the branched Quip4NSuc, the OPS contains D-Glc, D-GlcNAc, D-GalA, and two L-Rha residues, located in the main chain. The hexasaccharide repeating unit of the *P. mirabilis* O49 OPS is the largest among the *Proteus* O-antigens.

The *P. mirabilis* S1959 strain was classified to the O3-serogroup and its OPS chain is built up of pentasaccharide repeating units with an amide-linked lysine residue (Table I) (Ziółkowski *et al.*, 1997; Amano *et al.*, 1996). The polymer of the pentasaccharide repeating units of the O3-antigen is attached to a core oligosaccharide, characterized by the presence of D-galacturonic acid residues at both the terminal and inter-chain positions (Vinogradov *et al.*, 2000). By chemical mutation of the parent, smooth *P. mirabilis* S1959 (O3) strain, two mutants producing polysaccharide-truncated LPSs were isolated. The *P. mirabilis* R110 strain produces Ra-type LPS with a complete core oligosaccharide, but lacks the O-antigen (Vinogradov *et al.*, 2000). The *P. mirabilis* R45 strain contains a truncated core oligosaccharide (Re-type) LPS (Amano *et al.*, 1996; Vinogradov *et al.*, 2000).

One of the functions of the complement system in host defense is self-/non-self- recognition (Blatteis *et al.*, 2004). The LPSs and outer-membrane proteins (OMPs) located on the outer-membrane of Gram-negative bacteria are important targets for the bactericidal action of the complement system (Blatteis *et al.*, 2004). The structure of the O-specific side chain of LPS and any structural change or chemical modification of it play important roles in the resistance of bacterial cells to the lytic activity of complement (Lachowicz *et al.*, 1999).

The aim of this study was to compare the actions of two lipopolysaccharides from *P. mirabilis,* differing in the polysaccharide residue of the endotoxin, on properties of the plasma membrane of human red blood cells. The membrane lipid fluidity, physical state of proteins, and osmotic fragility were determined by electron paramagnetic resonance (EPR) spectroscopy. The oxidative stress in the red blood cells was estimated by measuring the level of thiobarbituric acid-reactive substances and carbonyl compounds. Human erythrocyte lysis mediated by rough and smooth *P. mirabilis* LPSs, specific antibodies, and complement was also shown.

Materials and Methods

Chemicals

The spin labels: 5-doxylstearic acid (5-DS), 12-doxylstearic acid (12-DS), 16-doxylstearic acids (16-DS), 4-maleimido-2,2,6,6-tetramethylpi-

peridine-1-oxyl (maleimide spin label, MSL), and 4-iodoacetamido-2,2,6,6-tetramethylpiperidine-1-oxyl (iodoacetamide spin label, ISL) were from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals of analytical grade were purchased from Polskie Odczynniki Chemiczne (Gliwice, Poland).

Bacterial strain, growth and isolation of the lipopolysaccharides

P. mirabilis O7 (PrK 15/57), O33 (PrK 59/57), O38 (PrK 64/57), O49 (PrK 75/57) were from the Czech National Collection of Type Cultures (Institute of Epidemiology and Microbiology, Prague). *P. mirabilis* O3a,3b (OXK, S1959) strain and its two Re- and Ra-type mutants R45 and R110, respectively, originated from the Institute of Microbiology and Immunology, University of Lodz, Poland. *P. mirabilis* strains were cultivated under aerobic conditions, in nutrient broth (BTL, Warsaw, Poland) using a fermenter (Chemap AG, Volketswil, Switzerland) under controlled conditions [37 °C, pH 7.4–7.6, $p(O_2)$ 75–85%]. The cells were harvested at the end of the logarithmic growth phase, centrifuged ($5000 \times g$, 30 min), washed with distilled water, and lyophilized. The smooth form of LPSs was isolated by the phenol/water procedure (Westphal and Jann, 1965) and R-types by the PCP method (Galanos *et al.*, 1969). LPSs were purified by treatment with DNase and RNase (Boehringer Mannheim, Germany) as described by Gmeiner (1997). The LPS preparations obtained were essentially free of nucleic acids and contained <2.5% proteins.

Degradation of the lipopolysaccharide, complement and lipid A preparation

The *P. mirabilis* LPS (90 mg) was hydrolyzed with 2% acid/water solution at 100 °C for 1.5 h, and the lipid A precipitate was collected by centrifugation at $13{,}000 \times g$.

Blood samples were taken from healthy donors by vein puncture and collected in dry vacutainer tubes. Then the samples were allowed to clot for about 1 h at room temperature; thereafter they were centrifuged at 4 °C. The serum was frozen in liquid nitrogen and stored in 0.2-ml aliquots at –70 °C until tested (Andersson *et al.*, 2005).

Hemolysis assay

Blood was obtained from healthy young (25–30 years) male donors. The red blood cells (RBCs) were washed three times and resuspended in sterile cold phosphate-buffered saline (PBS) to give about $15{\cdot}10^6$ cells per ml and further processed. The erythrocytes were incubated with O7, O38, O49, S1959, R45, or R110 LPS of *P. mirabilis* at 2 μg/ml, 10 μg/ml, and 50 μg/ml for 18 h at room temperature, washed three times in sterile PBS buffer, followed by incubation for 1 h at 37 °C with respective rabbit anti-*P. mirabilis* antibodies. In the next step, RBCs were washed three times in sterile PBS, and then erythrocytes were incubated with 5 × diluted human serum in PBS (as a source of complement) for 2 h at 37 °C. After centrifugation of the non-hemolysed erythrocytes (100 g, 15 min), the absorbance of the released hemoglobin in the wavelength range 400–600 nm was measured. The percentage of immunohemolysis was determined. Positive control (100% hemolysis) was estimated as The amount of hemoglobin released from $15 \cdot 10^6$ RBCs after 10 min of incubation with water was estimated as positive control (100% hemolysis).

Isolation of erythrocytes and ghosts for EPR measurements

Human blood was collected from healthy subjects attending the outpatient clinic of a local hospital. Heparin was used as an anticoagulant. Blood was centrifuged, and plasma and white cells were removed. The erythrocytes were washed three times with PBS, pH 7.4, at 4 °C. The erythrocytes were suspended in PBS at a hematocrit of 50%. The erythrocyte ghosts were prepared after RBC treatment with LPS by the modified method of Dodge *et al.* (1963) by hypotonic lysis with 20 mmol/l sodium phosphate buffer, pH 7.4, at 4 °C. The ghosts were centrifuged and washed with 20 mmol/l, 10 mmol/l, and 5 mmol/l phosphate buffer, pH 7.4.

Incubation of erythrocytes with lipopolysaccharides

Erythrocytes at a hematocrit of 50% were incubated with increasing concentrations of O33 and O49 LPSs (0.1, 0.5, 1 mg/ml) for 1 h at 37 °C and washed with 10 mmol/l phosphate buffer. The concentrations in the experiments with lipid A were 0.05, 0.25, and 0.5 mg/ml.

Spin labeling of erythrocytes and ghosts

Erythrocytes were labeled with three spin-labeled fatty acids (5-DS, 12-DS, and 16-DS) by introducing a stock solution of doxyl derivatives in ethanol into the erythrocyte suspension and incubation for 0.5 h at room temperature. The final ethanol content in the erythrocyte suspension did not exceed 0.05% (v/v). Isolated ghosts (protein concentration: approx. 3 mg/ml) were labeled by adding 2 μl of an ethanol solution of 0.1 mol/l MSL or ISL, respectively, into 1 ml of the ghost suspension. The unbound spin label was removed by several washings with cold phosphate buffer until the EPR signal in the supernatant disappeared. All procedures were performed at 4 °C. EPR spectra were recorded on a Bruker ESP-300E (X-band) spectrometer operating at a microwave frequency of 9.73 GHz using the following instrument settings: centre field set at 3480 G, range 80 G, with a 100-Hz modulation frequency and a modulation amplitude of 1 G. All measurements were made at room temperature.

Osmotic fragility measurements

Osmotic fragility was measured by introducing erythrocyte suspensions to solutions containing decreasing (<0.155 mmol/l) concentrations of sodium chloride. The absorbance of the supernatant was measured at 575 nm. The experiments were carried out in a Pharmacia-LKB spectrophotometer. The extent of hemolysis was calculated from the equation $H = (A_x - A_c)/(A_{100} - A_c)$, where H denotes the extent of hemolysis, A_x the absorbance of the sample, A_c the absorbance of the control, and A_{100} the absorbance after complete hemolysis.

Determination of lipid peroxidation

The level of lipid peroxidation was determined according a modified method of Buege using thiobarbituric acid (TBA) (Buege and Aust, 1978). After incubation with LPS the erythrocyte suspension was washed with PBS solution and centrifuged. Then 0.375% (w/v) TBA and 15% (w/v) TCA in 0.2 M HCl were added to 1 ml of the erythrocyte suspension at a hematocrit of 1%. Then the samples were heated for 20 min at 100 °C, cooled, and centrifuged. The absorbance of the supernatant was measured at 532 nm. The concentration of TBARS was calculated in μM.

The level of oxidative modifications of erythrocyte components was measured by monitoring the carbonyl group formation. Carbonyl groups in erythrocytes were estimated according Levine *et al.* (1990). Suspensions of 10 μl of erythrocytes (hematocrit: 50%), after incubation with the LPSs, were added to 1 ml of 2,4-dinitrophenylhydrazine (DNPH, 20 mM solution in 2.5 M HCl), which is a specific reagent for carbonyl groups. After 1 h of incubation at room temperature the proteins were precipitated with 1 ml of 20% TCA. Then the samples were centrifuged, and the precipitate was washed three times with an 1:1 mixture of ethyl acetate and ethanol. The residue was dissolved in 1 ml of guanidine hydrochloride (6 M). The level of the carbonyl groups was measured spectrophotometrically at 370 nm. The carbonyl concentration was calculated using a millimolar absorption coefficient of 21.01 mmol/cm and expressed as μmol/gHb.

Statistical analysis

Statistical analysis included the calculation of means ± SD. The normal distribution of data was confirmed using the Shapiro-Wilk test. The significance of differences was estimated by one-way ANOVA and Tukey's test for multiple comparisons.

Results

Spin labeling

Membrane lipid fluidity and the conformation of membrane proteins were measured using a spin-labeling method. The fluidity of the lipids was estimated using three spin-labeled stearic acids of doxyl derivatives: 5-doxylstearic acid, 12-doxylstearic acid, and 16-doxylstearic acid. All the applied labels locate to different depths of the lipid layer and provide information on the ordering of membrane phospholipid fatty acyl mobility. The ratio of h_{+1}/h_0 (where h_{+1} and h_0 are the heights of low-field and middle-field lines of the spectra, respectively) was calculated and served as a semi-quantitative measure of acyl chain flexibility corresponding to lipid bilayer fluidity (Stuhn-Sekalec and Stanacev, 1978). A significant increase in the h_{+1}/h_0 ratio was observed for 5-DS upon treatment of erythrocytes with O49 LPS at all concentrations

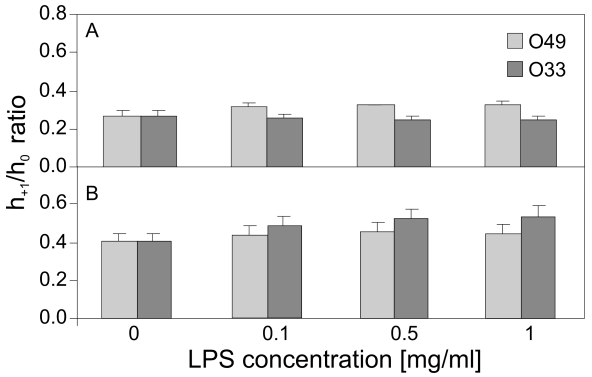

Fig. 1. Membrane lipid fluidity (expressed as the h_{+1}/h_0 ratio) in LPS-treated red blood cells monitored by (A) 5-DS (LPS O33: control vs. 0.1 mg/ml, $p < 0.01$; control vs. 0.5 mg/ml, $p < 0.001$; control vs. 1 mg/ml, $p < 0.005$; LPS O49: NS, $n = 5$) or (B) 12-DS (LPS O33: NS; LPS O49: control vs. 0.1 mg/ml, $p < 0.01$; control vs. 0.5 mg/ml, $p < 0.001$; control vs. 1 mg/ml, $p < 0.005$; $n = 5$).

(Fig. 1A). In the case of LPS O49, a significant increase was observed only for 5-DS but not for 12-DS. The increase in the h_{+1}/h_0 ratio for 5-DS reflects a rise in membrane lipid fluidity in the polar region of the lipid bilayer (changes were approx. 20% vs. a higher concentration of LPS). Smaller alterations in the membrane fluidity upon LPS O49 treatment of RBCs were observed in the deeper region of the lipid bilayer, as indicated by 12-DS (approx. 10%), but for LPS O33 the changes were much more profound (Fig. 1B). On the other hand, none of both lipopolysaccharides induced changes in the deeper region of the lipid bilayer, as indicated by 16-DS (results not shown).

The effect of lipid A on erythrocytes was also studied. All the applied concentrations of the compounds did not change the membrane lipid fluidity, as indicated by 5-, 12-, and 16-DS.

The physical state of the plasma membrane was determined using two spin labels (MSL and ISL). Both labels form covalent bonds with thiol groups of the membrane proteins (Berliner, 1983). These labels are frequently used for monitoring conformational changes in protein domains. Maleimide attached to an intact isolated human erythrocyte membrane yields a characteristic narrow-line (h_w), coming from weakly immobilized, and a broad-line (h_s), from strongly immobilized components, in the EPR spectrum. The h_w/h_s ratio is a very sensitive measure of the physical state of membrane proteins (Fung, 1983). Maleimide attached to membrane proteins did not indicate changes in the h_w/h_s ratio in both LPS-treated erythrocytes (data not shown).

In contrast, ISL attached to a human erythrocyte membrane yields a simple triplet spectrum. For the estimation of ISL mobility, the ratio of h_{+1}/h_0 was calculated. The ratio of h_{+1}/h_0 reflects alterations in membrane protein mobility. An increase in the ratio h_{+1}/h_0 of ISL was observed only for the highest concentration of LPS O33, but not for LPS O49. Other LPS concentrations did not change the mobility of this label attached to the membrane proteins.

Osmotic fragility assay

None of the lipopolysaccharides influenced the osmotic fragility of RBCs. Similar results were obtained for the osmotic fragility of erythrocytes using lipid A (results not shown).

Indicators of oxidative stress

The level of TBARS was measured upon LPS treatment of erythrocytes. Both LPS O49 and O33 in all concentrations did not influence the level of TBARS in comparison with untreated cells (data not shown). On the other hand, both higher concentrations (0.5 and 1 mg/ml) of O33 LPS and the highest concentration of O49 LPS induced increases in the level of carbonyl compounds in erythrocytes (Fig. 2).

Hemolysis of human erythrocytes in the presence of LPSs, antibodies, and complement

The activated antibody-dependent complement activation cascades of the *P. mirabilis* LPSs may affect human by-stander erythrocytes. To test this, RBCs were incubated in the presence of *P. mirabilis* LPSs, anti-LPS antibodies, and human serum as

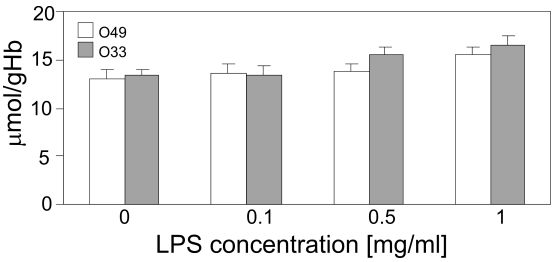

Fig. 2. Level of carbonyl compounds in control and LPS-treated red blood cells (LPS O33: control vs. 1 mg/ml, $p < 0.005$; LPS O49: control vs. 0.5 mg/ml, $p < 0.001$; control vs. 1 mg/ml, $p < 0.005$; $n = 5$).

Table II. Hemolysis of human erythrocytes incubated with S1959, R45, R110, O7, O38 and O49 *P. mirabilis* LPSs in the presence of anti-LPS antibodies and complement. The value of hemolysis was measured at 420 nm and 540 nm of the hemoglobin spectrum, and percentage of hemolysis was estimated in relation to the value of the positive control (100% of hemolysis).

LPS	Concentration [μg/ml]	Percentage at 420 nm (%)		Percentage at 540 nm (%)	
		–	+	–	+
S1959	2	5.10	6.06	3.79	3.56
	10	6.13	6.89	5.69	5.21
	50	2.17	8.91	4.98	8.06
R45	2	2.26	2.26	1.42	1.45
	10	3.42	14.63	0.71	5.17
	50	1.06	8.15	0.23	2.84
R110	2	3.42	**7.39**	0.71	**7.82**
	10	2.21	**34.36**	0.47	**33.89**
	50	2.86	**39.73**	2.84	**38.15**
O7	2	1.105	2.928	0.784	1.921
	10	1.657	3.845	1.765	1.961
	50	2.210	4.226	1.510	1.961
O38	2	2.420	3.077	1.961	2.686
	10	2.762	2.987	2.921	3.823
	50	2.972	2.597	3.921	4.352
O49	2	0.552	1.934	1.961	3.412
	10	2.762	3.597	1.961	4.608
	50	3.529	4.287	2.921	4.706

+, In the presence of complement; –, without complement, control.

the source of complement. The presence of LPSs causes erythrocyte lysis, detected by immunofluorescence. The erythrocyte cell is encircled by fluorescence-labeled antibodies reacting with R110 LPS (data not shown).

The amount of hemoglobin released by LPSs, complement, and the presence of anti-LPS antibodies was determined by a spectophotometric method (Table II). The most effective hemolysis was observed with R110 LPS. In a dose-dependent manner, up to 39% of the erythrocytes were hemolysed. Interestingly, the smooth O7, O38, O49, and O3 (S1959) and rough R45 LPSs have a hemolytically weak activity. This may indicate that the core oligosaccharide of R110 LPS plays an important role in the complement-mediated hemolysis of human erythrocytes.

Discussion

The alterations in the plasma membrane of erythrocytes, *e.g.* lipid fluidity, conformation state of membrane proteins, and osmotic fragility, and indicators of oxidative stress in erythrocytes in response to increasing concentrations of the bacterial smooth lipopolysaccharides O33 and O49 isolated from the *Proteus mirabilis* strain were examined. Both LPSs induced an increase in membrane lipid fluidity. LPS O49 induced higher changes in lipid fluidity in the polar region of the lipid bilayer and weaker changes in the deeper region. On the other hand LPS O33 induced slight alterations in the fluidity in the polar region but much bigger in the hydrophobic core (as indicated by 12-DS). All concentrations of LPS fluidized lipids in this region of the lipid bilayer. Both endotoxins did not influence the fluidity in the deeper region of the lipid bilayer measured by 16-DS.

In earlier studies, using 5-DS, it was shown that LPS from *Escherichia coli* did not change the membrane fluidity of rat erythrocytes (Butterfield *et al.*, 1994). On the other hand, in another study a decrease in membrane fluidity was observed using the same endotoxin, but at a higher concentration (Hino *et al.*, 1993). When studying the membrane lipid fluidity using 5-DS, we found a significant increase in membrane lipid motion for all concentrations of O49 LPS.

Recently it has been reported that higher concentrations of the endotoxin *Proteus mirabilis* S1959 led to an increase RBC fluidity in the deeper region of the lipid bilayer (Poschl *et al.*, 2003). This is in accordance with results obtained for RBCs treated with O33 LPS.

For the explanation of the role of action of lipid A on membrane fluidity, isolated lipid A from LPS

was applied. The LPS particles from Gram-negative bacteria contain a lipid part termed lipid A. It has been reported that lipid A is able to mimic the biological effect of LPS and represents a toxic centre of LPS (Rietschel *et al.*, 1994). Lipid A, a hydrophobic molecule, may directly interact with the membrane lipids of erythrocytes. It has been shown that the red blood cells of septic patients can bind endotoxins (Kaca *et al.*, 1998).

However, in our experiments we found that isolated lipid A did not influence plasma membrane properties. We did not observe changes in membrane fluidity, as indicated by the three spin-labeled fatty acids, as well as in the osmotic fragility of erythrocytes. It is possible that lipid A forms micelles with the polar group located outside. In this model the micelles are pushed away from the surface of the erythrocytes. We recently described a similar effect of hydrophobic interaction of antibiotics with *P. vulgaris* O25 LPS (Arabski *et al.*, 2007).

Using two covalently bound spin labels we aimed to investigate whether both O33 and O49 LPSs cchange the mobility of peripheral and cytoskeleton membrane proteins. A tendency to increase the mobility of the covalently bound ISL attached to membrane proteins upon LPS treatment of RBCs was observed. The significant increase in the motion of spin label residue was found for higher concentrations of LPS O33. However, this label reacts with the peripheral proteins. On the other hand, we did not observe changes in the mobility of the MSL. This label reacts with the internal surface of the RBC membrane, mainly with the spectrin-actin complex (Fung, 1983), and our results showed that this region was inaccessible for LPS particles. These results showed that both LPS particles interact predominantly with the external surface of the plasma protein. It has been shown that the action of LPS on isolated membranes was more profound when LPS had much easier access to the internal surface of the RBC membranes (Gwoździński *et al.*, 2003).

Another piece of evidence that LPS did not change the integrity of the lipid bilayer was the lack of alterations in the osmotic fragility of the RBCs.

It is possible that the hydrophobic part of LPS, lipid A, interacts or partially intercalate with/to phospholipids from the outer surface of the plasma membrane. The hydrophobic incorporation of LPS into the phospholipid liposomes was found only upon long-term incubation at physiological temperature or in the presence of divalent cations (Schromm *et al.*, 1995).

The observed differences in the interaction of both LPSs with erythrocyte surface plasma membranes might be due to the significant differences in the LPS structures. *P. mirabilis* O33 LPS is characterized by two phosphate residues and a highly negative charge in contrast to O49 LPS.

The modulatory effects of polysaccharides on lipid A may depend on the peculiar, unique structures of O-polysaccharides.

Changes in membrane lipid fluidity may be a result of the interaction of the hydrophobic part of LPS with the membrane and lead to changes in lipid-lipid or lipid-protein interactions in the lipid bilayer of the membrane. Hydrophobic interactions between the lipid component of LPS were confirmed in the artificial mono- and bilayer of phospholipid membranes (Schromm *et al.*, 1995). In this study we showed that the highest concentration of LPS, especially of LPS O33, increases the content of carbonyls compounds, which reflect protein oxidation. Changes in protein mobility can result in action of LPS particles or oxidation of peripheral proteins. It has been shown that LPS can produce reactive oxygen species in erythrocytes (Hino *et al.*, 1993). On the other hand we did not find a higher TBARS level upon erythrocyte treatment with LPS. It has been reported that membrane proteins are more sensitive to oxidation than lipids (Gwoździński, 1991).

The interaction of *P. mirabilis* LPSs with the human erythrocyte membrane was observed by a complement-mediated immuno-hemolysis test. Only a small number of RBCs was lysed by a series of smooth form LPSs alone, in the presence of anti-LPS antibodies, or complement proteins. The only exception was the Ra-type R110 LPS, which was hemolytically highly active. This may indicate that complement-mediated immuno-hemolysis is inhibited by the presence of long O-specific chains of LPSs. The complete core oligosaccharide of R110 LPS seems to facilitate RBC lysis in the presence of immuno-complexes and complement proteins. The smooth form of lipopolysaccharides activated human complement by the classical, antibody-dependent pathway. The membrane attack complexes (MACs) are not formed or formed in the fluid phase at long distances from the plasma membrane of erythrocytes. The very different O-polysaccharide structures do not play a role in

such activities. The few percent of lysis of erythrocytes in the presence of S LPSs may have resulted from incomplete substitution of the core oligosaccharide by long O-specific polysaccharides. The presence of the R-type of LPSs in the *Proteus* S form was reported (Poschl *et al.*, 2003; Kaca *et al.*, 1998). The mutant Ra-type R110 LPS is depleted of long O-specific chains. This causes MACs to be formed in close proximity to the plasma membrane of erythrocytes. The MAC intercalated to the plasma membranes of the erythrocytes, formed channels, and disruption of the plasma membrane occurred. That phenomenon resulted in an increase of hemoglobin release from erythrocytes treated with *P. mirabilis* LPSs, anti-LPS antibodies, and human serum as a complement source.

It is worth noting that the doses of *Proteus* LPSs used in the complement-mediated immuno-hemolysis test were 100 times lower than that used in EPR methods. However, this amount of LPS was sufficient for complement-enhanced lysis of RBCs.

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