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Effects of Chitosan on the Biological Properties of Gram-Negative Bacteria Endotoxins

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The destructive action of Gram-negative bacteria lipopolysaccharides and their complexes with chitosan, a natural polycation, was compared *in vivo* and *in vitro*. Lipopolysaccharides in complex with chitosan modified the biological properties of endotoxin, among other things, reducing its toxic and aggregation effects.

Key Words: *platelet aggregation; lipopolysaccharides*

Studies of the pathogenesis of infectious diseases caused by Gram-negative bacteria have revealed a broad spectrum of destructive action of toxins in the patient's organism: changes in the diameter and rate of contraction of microcirculatory vessels, vascular hypersensitization to adrenalin, necrosis of vascular endothelium with denudation of the basal membranes, and disorders in the hemocoagulation system involving primarily the blood platelets [1-3]. At the same time, the potentialities of lipopolysaccharides (LPS) - components of the bacterial wall - as adjuvants, mitogens, lymphokine synthesis inductors, and antitumor and radioprotective agents in medicine and biotechnology have been widely studied in recent years. However, the extremely toxic properties of LPS prevent their use in medical practice, and therefore agents are needed which will, on the one hand, inhibit the injurious action of endotoxins in the patient's organism and, on the other, will be low-toxic derivatives of endotoxins or their synthetic analogs retaining their immunomodulating, antitumor, and antiseptic activities. Such studies may be developed along two lines: the preparation of endotoxin complexes with polycations of natural origin and the use of polycations and their complexes in medicine and biotechnology.

One of the most prevalent cations isolated from natural sources is chitosan (1,4-polyglucosamine). The ready availability and relative biological inertness of chitosan lend themselves to the preparation of various biological agents.

This research was aimed at designing a chitosan complex with LPS of Gram-negative bacteria and performing a comparative experimental study of their biological activities.

MATERIALS AND METHODS

Yersinia pseudotuberculosis LPS were extracted from a bacterial mass as described previously [8] and purified from nucleic acids by ultracentrifugation at 150,000 g. The resultant LPS contained less than 1% protein. LPS of *Escherichia coli*, serotype

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0111:B4, was from Difco. *Y. enterocolitica* protein-LPS complex (PLPSC) was prepared as reported elsewhere [7]. Chitosan was prepared by alkaline treatment of chitin after Horowitz *et al.* [6]. The conditions of formation of the LPS-chitosan complex were studied by sedimentation methods. The LPS-chitosan complex was prepared by adding 1 ml of chitosan solution in concentrations ascending from 1 to 10 mg/ml to 1 ml of LPS solution (1 mg/ml) followed by 36-hour incubation at 37°C. The LPS concentration in the solution was constant. Samples of LPS and LPS-chitosan complex were dissolved in 10 ml of cesium chloride solution at a density of 1.4 mg/ml.

The toxicities of *Y. pseudotuberculosis* and *E. coli* LPS and their complexes with chitosan were studied *in vivo* on outbred white mice and C57Bl mice weighing approximately 20 g.

The effects of chitosan, LPS and PLPSC of Gram-negative bacteria and their complexes with chitosan on platelet aggregation activity were recorded using a Russian-made aggregometer as described previously [5]. Aggregation was induced with the use of adenosine diphosphate (ADP, 2×10^{-5} M, *Y. pseudotuberculosis* and *E. coli* LPS, *Y. enterocolitica* and *Y. pseudotuberculosis* PLPSC, chitosan, and LPS-chitosan complex. In the control normal saline was added to platelet-rich plasma instead of the test substances. The degree of irreversible platelet aggregation caused by ADP alone was the control value, 100%. After addition of the test substance the process of aggregation was recorded before the curve reached a plateau and only then was ADP added.

The shape of the platelets and the state of their plasma membranes were studied under a Philips scanning electron microscope.

The results were statistically processed by estimating the confidence coefficient.

RESULTS

Use of the accelerated sedimentation method made it possible to detect the formation of chitosan complexes with Gram-negative bacteria LPS. LPS were found to be completely saturated with chitosan at a concentration of chitosan five times surpassing the concentration of *Y. pseudotuberculosis* LPS and seven times surpassing the concentration of *E. coli* LPS. The complexes were stable and did not dissociate in a solution of high ionic strength. Fluorescent derivatives of chitosan were used in studies of complex formation at low concentrations of the agent.

Hence, LPS from various sources interact with chitosan in aqueous solutions to form complexes of different stoichiometry. The stoichiometry of a complex depends to a great extent on the concentration of the reagents and varies at weight ratios from 5:1 to 1:5.

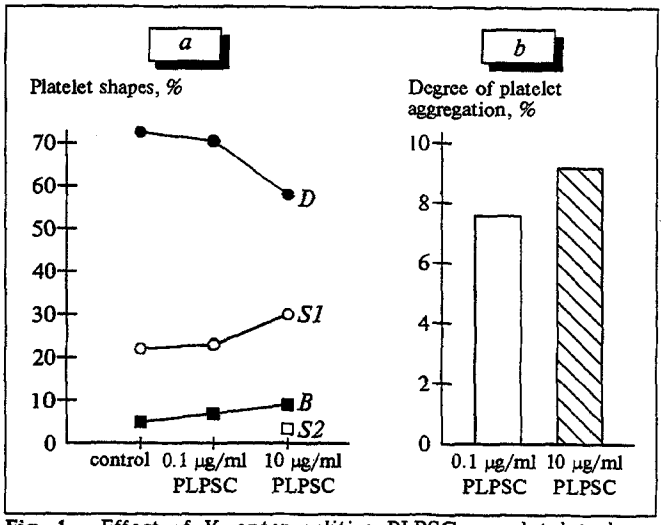

Fig. 1. Effect of *Y. enterocolitica* PLPSC on platelet shape (*a*) and *in vitro* aggregation (*b*). Discoid (*D*), bipolar (*B*), and spheroidal (*S1* and *S2*) platelets.

Assuming that LPS complexes with chitosan can modify the biological characteristics of the endotoxin, we decided to study some of them. Toxicity is one of the most salient characteristics of LPS. Since chitosan seems to bind LPS through charged polycation groups and a site of endotoxin core lipid A, it was this very activity that was expected to change. For this reason we studied the toxicity of the complex with chitosan for LPS from *E. coli* and *Y. pseudotuberculosis.*

The results indicate a lower toxicity of LPS-chitosan complexes in weight ratios of 1:1 and, all

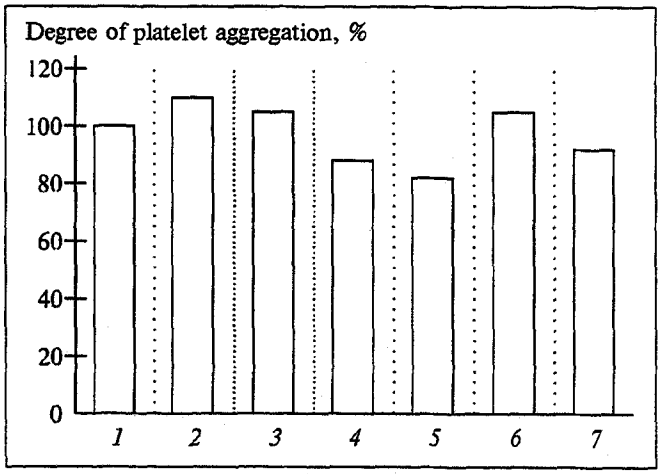

Fig. 2. Effect of different doses of *E. coli* LPS, chitosan, and LPS–chitosan complex on platelet aggregability. ADP–induced aggregation (*1*, control); after 5–min incubation with *E. coli* LPS in doses of 2 (*2*) and 20 (*3*) μg/ml, with chitosan in doses of 14 (*4*) and 140 (*5*) μg/ml, and with LPS–chitosan complex in doses of 16 (*6*) and 160 (*7*) μg/ml.

TABLE 1. Toxicity of *E. coli* LPS and Its Complexes with Chitosan upon Injection to Outbred and C57Bl Mice in the Presence of Galactosamine

Agent	LPS concentration, μg/mouse	Mortality	
		outbred white mice, dead/total	C57Bl mice, dead/total
LPS	0.4	6/6	6/6
	0.2	6/6	6/6
	0.1	6/6	6/6
	0.05	3/6	5/6
	0.025	3/6	6/6
	0.0125	1/6	4/6
LPS–chitosan complex (5:1)	0.4	6/6	5/6
	0.2	3/6	5/6
	0.1	1/6	6/6
	0.05	0/6	3/6
	0.025	0/6	1/6
LPS–chitosan complex (1:1)	0.1	2/6	–
	0.025	0/6	–
LPS–chitosan complex (1:5)	0.1	0/6	–
	0.025	0/6	–

the more so, 1:5, in comparison with *E. coli* LPS (Table 1).

For outbred and C57Bl mice the toxicity of LPS in the complex was 4-8 times lower. C57Bl mice proved to be more sensitive to the toxic action, but the regularity was true for them as well.

The toxicity of *Y. pseudotuberculosis* LPS and its complex with chitosan in a 5:1 ratio was studied for their administration in parallel with actinomycin D (Table 2). The use of such a mode of injection enabled the toxicity of LPS in the complex to be reduced 20-fold. Hence, we may consider it proven that when they enter into complexes with chitosan, LPS lose some of their toxicity. This may be due to shielding of the core lipid A-toxophore group of the endotoxin by a chitosan molecule, or to alteration of the charge and physicochemical parameters of its entire molecule.

A recent increase in the incidence of yersiniosis prompted us to investigate the effects of *Y. enterocolitica* and *Y. pseudotuberculosis* PLPSC and of *Y. pseudotuberculosis* and *E. coli* LPS and complexes thereof on the functional characteristics of human platelets [4,9].

For a study of the role of endotoxin in the pathogenesis of the disease caused by *Y. enterocolitica, Y. enterocolitica* PLPSC was added to platelet-rich plasma of normal subjects (10 μg/ml) *in vitro*. PLPSC induced platelet aggregation of 9.2±1.0% *vs.* the control. After the aggregatogram reached a plateau, ADP was added to the cuvette. This caused a second wave of aggregation, reaching 100%. Hence, *Yersinia* PLPSC is an inductor of platelet aggregation.

As we know, the influence of PLPSC on platelet shape underlies the above mechanism. The count of disklike forms (resting cells) was found to be reduced on account of their transformation into activated ones - spheroidal. Moreover, an increase of the PLPSC concentration to 10 μg/ml led to the appearance of S2 forms which do not occur in health. In addition, an increase of the PLPSC concentration (0.1-10 μg/ml) was associated with a simultaneous intensification of platelet aggregation

TABLE 2. Toxicity of *Y. pseudotuberculosis* LPS and Its Complex with Chitosan (1:5) upon Injection to Outbred White Mice in the Presence of Actinomycin D

Agent	LPS concentration, μg/mouse	Number of mice	Mortality, dead/total	Mortality, %
Y. pseudotuberculosis LPS	2.4	6	5/6	83
	1.2	6	3/6	50
	0.6	6	1/6	17
LPS–chitosan complex (1:5)	50.0	6	5/6	83
	25.0	6	3/6	50
	12.5	6	1/6	17

and an increase in the count of spheroidal cells (Fig. 1).

The injurious action of *Y. pseudotuberculosis* on blood cells was studied using *Y. pseudotuberculosis* LPS and PLPSC. LPS of *Y. pseudotuberculosis* in the tested doses (10 and 50 μg) did not cause platelet aggregation and did not influence the total aggregation upon subsequent addition of ADP.

Subthreshold doses of *Y. pseudotuberculosis* PLPSC (10 and 20 μg) were ineffective. At the same time, in contrast to LPS, addition of 50 μg of PLPSC to donor platelets caused a reduction of the degree of ADP-induced platelet aggregation (after ADP preincubation with toxin) and of the total degree of aggregation for the consecutive addition of the toxin and ADP.

Direct exposure to *E. coli* LPS did not cause an appreciable aggregation of platelets, but noticeably changed their response to subsequent addition of ADP. Five-minute incubation of donor platelets with *E. coli* LPS in a dose of 2 μg/ml led to activation of the cells. This was manifested in an 11% ($p<0.001$) increase of the degree of aggregation under the effect of the standard ADP inductor in a dose of 2×10^{-5} M and in a 3% increase with an ADP dose of 20 μg/ml (Fig. 2). The less pronounced effect of the high LPS dose may be due to the appearance of numerous refractory cells incapable of aggregation.

Hence, our studies demonstrated that *Y. pseudotuberculosis* LPS and PLPSC, *Y. enterocolitica* PLPSC, and *E. coli* LPS are platelet activation inductors, their effects on the platelets being dose- and time-dependent.

The next series of experiments was devoted to the effects of chitosan and its complexes with *E. coli* LPS. Chitosan was found to suppress the action of ADP. Five-minute incubation of platelets with chitosan in a dose of 14 μg/ml reduced the degree of ADP-induced aggregation by 11% ($p<0.01$), while a chitosan dose of 140 μg/ml reduced it by 18% ($p<0.05$, Fig. 2).

A study of the effects of an LPS-chitosan complex under the same conditions revealed a reliable decrease of platelet aggregability - by 5 and 8%, respectively, at concentrations of 16 and 160 mg/ml in comparison with the total aggregation induced by LPS and ADP (Fig. 2).

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