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Experimental uveitis induced by different uniform salts of enterobacterial lipopolysaccharides

Eksperymentalne zapalenie błony naczyniowej wywołane przez jednorodne sole lipopolisacharydów enterobakteryjnych

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Background: Injection of lipopolysaccharides/LPS/, the major component of the outer membrane of gram-negative bacteria, can induce inflammation in the eyes of susceptible animals.The LPS-induce ocular inflammation is termed endotoxin-induced uveitis/EIU/and is characterized by iris hyperemia, miosis, a rise in aqueous humor protein, and inflammatory cell infiltration into the anterior uvea and aqueous humor. Biological activities of endotoxin depend also on its molecular weight.

Objectives: The aim of our study was to find the effect of different aggregation forms on the clinical and histopathological characteristics of the EIU.

Materials/Methods: Lipopolysaccharides were electrodialyzed and neutralized by adding: triethylomine, sodium hydroxide, or calcium hydroxide. EIU was produced in Lewis rats by footpad injection of different enterobacterial LPS. Their eyes were examined for clinical signs of inflammation in slit lamp, protein and cells were measured in the aqueous humor.

Results/ Conclusions: The correlation between the physical parameters and biological activity is discussed. Our results have shown recently that monomeric form of endotoxin is more active than an aggregated form in induction of experimental uveitis.

Key words: **endotoxin • lipopolysaccharide • experimental uveitis**

Streszczenie

Iniekcja lipopolisacharydów (LPS), będących jednym z głównych składników ścian bakterii Gram-ujemnych, może wywołać odczyn zapalny w oczach podatnych na nie zwierząt. Do zapaleń wywoływanych przez lipopolisacharydy bakteryjne należy tzw. zapalenie błony naczyniowej wywoływane przez endotoksyny bakteryjne (EIU– endotoxin induced uveitis). Aktywność biologiczna endotoksyn zależy także od ich masy molekularnej. W badaniach szukaliśmy wpływu różnych form agregacyjnych tych substancji na kliniczne oraz histopatologiczne aspekty EIU.

Lipopolisacharydy były poddawane elektrolizie, a następnie neutralizowane przez dodanie trietyloamin, wodorotlenku sodu oraz wodorotlenku wapna. EIU było wywoływane u szczurów Lewisa poprzez wstrzykiwanie do opuszki łapy zwierzęcia różnych typów lipopolisacharydów entero-

bakteryjnych. Ich oczy były następnie badane w lampie szczelinowej w celu diagnozowania objawów zapalenia. Obliczono także stężenie białka i komórek zapalnych w płynie komorowym.

Przedmiotem dyskusji jest związek pomiędzy parametrami fizycznymi a aktywnością biologiczną tych związków. Nasze wyniki pokazują, iż monomeryczne formy endotoksyny są bardziej aktywne od ich form agregacyjnych w wywoływaniu doświadczalnego zapalenia błony naczyniowej.

Słowa kluczowe: **endotoksyna • lipopolisacharydy • eksperymentalne zapalenie błony naczyniowej**

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Abbreviations: **LPS** – lipopolysaccharide; **EIU** – endotoxin-induced uveitis; **LBP** – lipopolysaccharide binding protein; **TNF** – tumor necrosis factor; **TEA** – triethylamine; **IL** – interleukin

Introduction

Endotoxin is a main component of the cell wall of gram-negative bacteria. Enterobacterial cell envelope is built of inner membrane composed of phospholipids and proteins, and outer membrane of very unique structure.

Endotoxin is a main component of outer membrane and presents a main surface antigen- O-antigen. Endotoxin is a lipopolisaccharide (LPS) essential for physical organization and function of outer membrane, and generally for bacterial growth and multiplication. Endotoxins are virulence factors and are targets for bacteriophages. They can bind antibodies and non – immunoglobulin serum factors, prevent complement activation and uptake of bacteria by phagocytes.

Endotoxins show also many activties affecting the host, an infected higher macroorganism. They are pyrogenic, and can activate complement, B-lymphocytes, granulocytes and mononuclear cells. Endotoxins show also immunostymulatory and adjuvant activities. They induced production of diffrent cytokins: interleukins 1, 2, 6, 8, tumor necrosis factor, interferon, and colony stimulating factor. Finally they are responsible for circulatory collapse, multiple organ failure resulting in a lethal outcome [15].

The mechanism of endotoxin action on macrophage of the host organism is as follows: free endotoxin released from killed bacteria react with the acute phase protein, a lipopolysaccharide binding protein (LBP) present in serum. Interaction of the LPS-LBP complex with CD 14 receptor on macrophages induces synthesis of tumor necrosis factor (TNF) and three different types of mediators: proteins, free oxygen radicals and lipids but when there is an overproduction of mediators in severe bacteremia the effects are harmful for the host [2,15,19].

Endotoxins are responsible for initiation of septic shock which increases the number of fatalities in Gram-negative bacteremia among hospital patients. The morality from septic shock is still high despite recent developments in antibiotic therapy because antibiotics are unable to decrease the level of free lipopolysaccharide in the blood stream.

The biomedical significance of endotoxins has stimulated research into their chemical nature and attempts at development of immunological and pharmaceutical strategies which could prevent the harmful effects of endotoxin [1]. Chemical studies were performed on pure endotoxin preparation gentely isolated from bacterial smooth and rough strains. It was found that endotoxins derived from different bacterial species have a common structure [5,10]. Endotoxin (LPS) consist of a polysaccharide and a covalently bound lipid termed lipid A. In LPS of wild type-(smooth or S-form) Enterobacteria the polysaccharide component consist of two regions which differ in the genetically determined biosynthesis and chemical structure: O-specific chain is a polymer of repeating oligosaccharide units which contain up to eight different sugar residues which are generally interlinked by glycosidic bonds [18].

The nature, sequence, type of linkages and substitution of monosaccharide residues is characteristic of a given LPS. Because of the diversity of constituents and their linkages an enormous variety of structures occur in O- specific polysaccharides. In contrast to O-specific chain the structural variability of the core within diffrent bacterial species is limited.

Lipid A is a covalently linked lipid component of LPS. The ketosidic linkage between Kdo and lipid A is acid labile and free lipid A can be released by treatment with mild acid. Lipid A consists of the phosphorylated B (1–10) glucosamine disaccharide and usually four moles of 3-hydroxymyristic acid. Two of them are amide linked and two form

esters in position 3 and 3'. Both amide and esterbound hydroxy fatty acids are partially substituted by saturated fatty acids. Structural variability of lipid A is rather low.

Hafnia alvei is a typical member of Enterobacteriaceae. Bacteria of this species are Gram-negative, motile, peritrichously flagellated, rarely encapsulated rods [3]. *H. alvei* is an opportunistic pathogen found in some incidences of nosocomial infections, and cases of septicemia caused by these bacteria have also been presented [19]. It is frequently encountered in pathological specimens that are in most cases found in mixed cultures and in the isolation of *H. alvei* from the same incidences of gastroenteritis has also been reported. The serotyping scheme of *H. alvei* includes 39 O-serotypes and some of these show cross-reactivities with the O-antigens of certain strains of *Salmonella*, *Escherichia coli*, *Enterobacter cloacae*, and *Citrobacter freundii* [1]. The structures of the O-specific polysaccharides from *H. alvei* strains ATCC 13337, 2, 38, 39, 1187, 1191, 1205, 1211 and 1192 have been elucidated [7].

The detailed structure of the core region and lipid A of any *H. alvei* lipopolysacchride (LPS) has not been determined yet, except for some data concerning a core hexasaccharide in strain 2 and a core trisaccharide in strains 32 and 1192 [6] and strains 2 and 1211 [8].

Since the lipid A, core region and the O-specific polysaccharide (PS) plays an important role in bacterial physiology and interaction with the host, it was of current interest to characterize the biological activity of the *H. alvei* strain 981 endotoxin.

The systemic injection of LPS is known to induce endotoxin-induced uveitis (EIU) in rats and rabbits [11]. It has been shown that endotoxin affects macrophages, B lymphocytes and T lymphocytes and induces the production of several cytokines [12,13,20].

Since the interavitreal injection of endotoxin, interleukin1 (IL-1), interleukin 6 (IL-6) or tumor necrosis factor (TNF) [13] also induces uveitis in rabbits, these cytokines are thought to play an important role in the pathogenesis of EIU. Nevertheless, the cause of inflammatory cell infiltration of the eye in endotoxin- induced uveitis remains unclear.

The aim of our studies was to test the biological activities of different uniform salts of enterobacterial lipopolysaccharide isolated from *Hafnia alvei* in experimental induced uveitis in rats.

Materials and Methods

The *H. alvei* strain 981 was obtained from the collection of the Pasteur Institute (Paris). LPS was prepared by phenol-water extraction of bacterial cell and purified by column chromatography on Sepharose 2B [7].

LPS was analyzed by SDS-PAGE [6] and the LPS bands were detected by silver staining.

A solution of lipopolysaccharide was subjected to electrodialysis to remove all cations and lipopolysaccharide was obtained in the free acid form. It was subsequently neutralized (pH 7.0) with the appropriate base in order to obtain the required salt form: triethylamine (TEA), sodium and calcium.

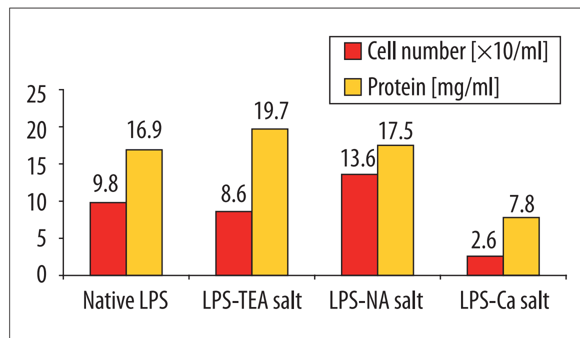

Figure 1. Cell number and protein concentration (mean values) in aqueous humor in experimental uveitis induced by different uniform salt forms of LPS.

Lipopolysaccharide preparations were dissolved in physiological salt solution at concentration 1 mg/ml by using ultrasonic water bath.

Introduction of EIU. 40 four-months-old male rats weighing 300–350 g were injected into a hind foot pad with 100 µg of LPS preparations.

The cell number and the protein concentration in the aqueous humor was measured in 4 groups using 10 rats in each group. Measuring of the cell number and the protein concentration were carried out as follows. Immediately after a rat had been sacrificed, the aqueous humor was carefully obtained without touching the iris using a 26-gauge needle under a microscope. A total volume of 5–10 µl of aqueous humor was obtained from the eyes of each animal.

The cell number was then counted using Bürke chamber. The protein concentration of the aqueous humor was measured using the bicinchoninic acid.

The degree of inflammation in anterior chamber was examined clinically using a slit lamp.

Results and Discussion

The data from experiments in which the uveitis was induced by three different uniform lipopolysaccharide salts are presented in Tables 1, 2, 3, 4 and on Figure 1.

Lipopolysaccharides usually contain inorganic cations like Na^+, K^+, Mg^{2+}, Ca^{2+} as well as low molecular weight basic amines like putrescine, spermine, spermidine and ethanolamine. They form salts with the acid groups of the LPS molecule (phosphate, pyrophoshate, carboxyl). LPS preparations in uniform salt forms prepared by electro-dialysis and neutralization with various bases differ in molecular weight and their anti-complementary activity. This has been shown for LPS form *Salmonella abotrus equi* and *S. typhimurium* S form [2,4]. Putrescine and sodium salt forms of LPS *Shigella sonnei* phase I showed high anticomplementary activity, while triethylamine form lost almost completely its ability to interact with com-

Table1. Uveitis induced by native-LPS.

LPS	Rat	Eye R/L	Slit lamp examination1	Aqueous humor cells $\times 10^6$/ml	protein mg/ml
Native-LPS	1	R	+	8.55	11.5
		L	+	13.70	13.5
	2	R	++++	1.50	15.0
		L	++	2.89	12.5
	3	R	+	8.20	11.5
		L	+	21.45	17.0
	4	R	+	7.25	15.0
		L	+	28.20	20.0
	5	R	++++	6.40	13.5
		L	++++	8.80	10.0
	6	R	–	8.00	20.0
		L	+	7.25	27.5
	7	R	+	4.95	20.0
		L	+	13.75	15.0
	8	R	+	–	–
		L	++++	24.00	20.0
	9	R	++++	1.10	18.5
		L	++++	5.55	15.0
	10	R	+	2.20	27.5
		L	+	21.70	18.5
Mean				9.77	16.92

[1] Inflammation score +, ++, +++, ++++; R – right, L – left.

Table 2. Uveitis induced by LPS-TEA salt.

LPS	Rat	Eye R/L	Slit lamp examination1	Aqueous humor cells $\times 10^6$/ml	protein mg/ml
LPS-TEA salt	1	R	–	3.60	18.5
		L	–	9.75	15.0
	2	R	+/–	0.15	7.5
		L	+	7.95	16.0
	3	R	–	5.35	28.5
		L	+	17.55	25.0
	4	R	++++	4.15	20.0
		L	++++	10.45	22.0
	5	R	++++	7.25	27.0
		L	++++	13.05	25.0
	6	R	++++	5.65	26.0
		L	++++	11.00	19.5
	7	R	+	4.65	21.5
		L	+	12.00	24.0
	8	R	+/–	2.80	10.0
		L	–	22.10	17.0
	9	R	+	7.10	6.5
		L	+	10.25	15.0
	10	R	+	14.55	27.0
		L	+	2.75	23.0
Mean				8.6	19.7

[1] Inflammation score +, ++, +++, ++++; R – right, L – left.

Table 3. Uveitis induced by LPS-Na^+ salt.

LPS	Rat	Eye R/L	Slit lamp examination1	Aqueous humor cells ×10⁶/ml	protein mg/ml
LPS-Na^+ salt	1	R	+	10.60	17.0
		L	+	11.00	27.5
	2	R	+	3.95	28.5
		L	+	4.15	18.0
	3	R	+	6.60	15.0
		L	+	28.65	13.5
	4	R	–	0.75	25.0
		L	–	14.15	15.0
	5	R	–	3.55	16.5
		L	+	9.50	13.0
	6	R	++++	13.30	10.0
		L	++++	17.20	16.5
	7	R	+	13.05	7.5
		L	++	19.80	14.5
	8	R	+	9.75	16.0
		L	+	17.40	21.0
	9	R	+	23.70	13.0
		L	++	17.80	32.0
	10	R	+	28.60	16.0
		L	+	19.25	7.5
Mean				13.63	17.2

[1] Inflammation score +, ++, +++, ++++; R – right, L – left.

Table 4. Uveitis induced by LPS-Ca^{++} salt.

LPS	Rat	Eye R/L	Slit lamp examination1	Aqueous humor cells ×10⁶/ml	protein mg/ml
LPS-Ca^{++} salt	1	R	+	4.30	2.0
		L	+	19.25	8.5
	2	R	+/–	0.95	3.5
		L	+/–	0.85	5.0
	3	R	+/–	0.65	14.0
		L	++	2.80	11.5
	4	R	+/–	0.60	17.5
		L	+	0.85	9.0
	5	R	+/–	0.55	4.0
		L	+/–	5.00	6.5
	6	R	+++	3.55	14.0
		L	++++	4.25	16.5
	7	R	+/–	0.16	3.5
		L	+	5.45	12.5
	8	R	+++	0.11	5.5
		L	+/–	1.27	4.0
	9	R	+/–	0.03	1.5
		L	+/–	0.00	1.0
	10	R	+++	0.74	5.5
		L	+++	0.60	11.0
Mean				2.59	7.82

[1] Inflammation score +, ++, +++, ++++; R – right, L – left.

plement [5]. Lipopolysaccharides interact with complement only when they are present in a state of high aggregation. In case of interferon induction by *Shigella sonnei* LPS the most active were low-molecular-weight triethylamine and ethanolamine salt forms. Highly aggregated putrescine form of LPS was hardly active or inactive. Lipid A ability to induced interferon depends also on its molecular weight.

In the present study, the aggregated form of LPS (Ca^{++}) was definitely less active in inducing inflammatory changes than monomeric forms (TEA, Na^{+}).

Our results are in agreement with results of Takayama et al. [17]. They have shown that monomeric form of endotoxin is more active than an aggregated form in activation of peritoneal macrophages.

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