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Immunotherapy in Gram-negative bacterial infections

Czesław Ługowski

Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, Czerska 12, 53–114 Wrocław, Poland

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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. Another approach to the treatment and prevention of septicaemia involves stimulation of an immune response against LPS. It was found that immunization with the core structures of endotoxin conjugated with proteins protected animals against infections and endotoxic shock. Anticonjugate sera are of great interest because they are directed against conserved parts of LPS and therefore could have cross-reactive and cross-protective properties with respect to many Gram-negative rods.

Endotoxin is a main component of the cell wall of Gram-negative bacteria. *Enterobacterial* cell envelope is built of an inner membrane composed of phospholipids and proteins, and an outer membrane of very unique structure. A periplasmic murein layer separates both membranes. Endotoxin is the main component of outer membrane and represents a main surface antigen — O-antigen. Endotoxin is a lipopolysaccharide (LPS)[1] 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 activities affecting the host, an infected higher macroorganism. They are pyrogenic, and can activate complement, B-lymphocytes, granulocytes and mononuclear cells. Endotoxins show also immunostimulatory and adjuvant activities. They induce production of different 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 [1].

The mechanism of endotoxin action on macrophage of the host organism is as follows: free endotoxins 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 CD14 receptor on macrophages induces synthesis of tumour necrosis factor (TNF) and three different types of mediators: proteins, free

[1]Abbreviations: Ab, antibody; Kdo, 2-keto-3-deoxyoctulosonic acid; LBP, lipopolysaccharide binding protein; LPS, lipopolysaccharide; OS core, oligosaccharide core; R, rough; Ra, *Salmonella* oligosaccharide core chemotype (complete core); R1-R4, K-12, *Escherichia coli* oligosaccharide core chemotypes; Re, *Salmonella* oligosaccharide core chemotype containing Kdo region only; SDS, sodium dodecyl sulphate; TT, tetanus toxoid.

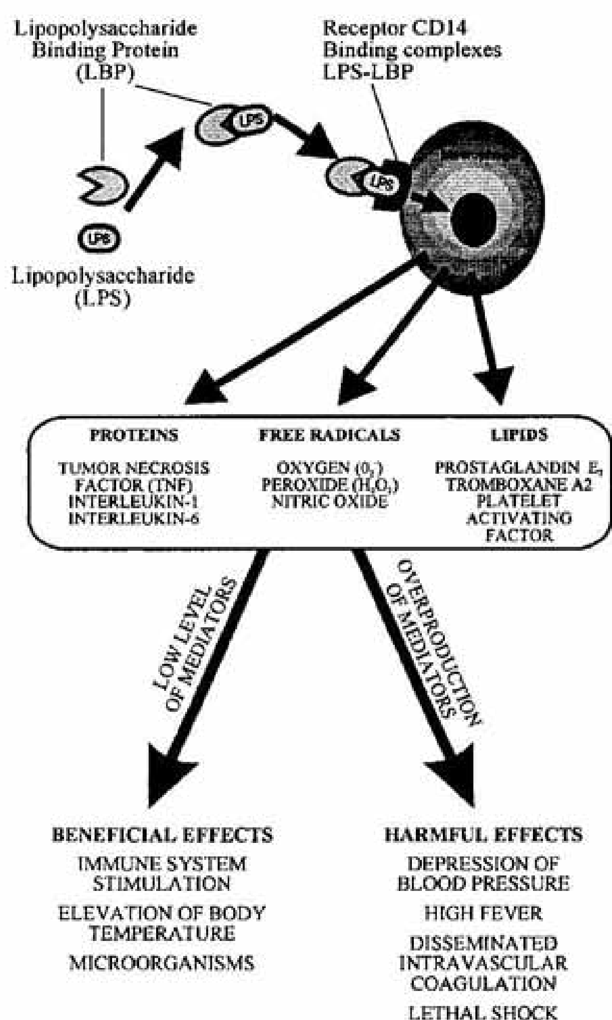

Fig. 1. Endotoxin induced macrophage stimulation.

oxygen radicals and lipids (Fig. 1). When the level of mediators is low they can induce beneficial effects for the host, but when there is an overproduction of mediators in severe bacteremia the effects are harmful for the host [2].

Lipopolysaccharides are not intrinsically toxic. It is the self-disintegrating response of the host organism to endotoxin which makes these molecules poisonous.

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. Chemical studies were performed on pure endotoxin preparation gently isolated from bacterial smooth and rough strains. It was found that endotoxins derived from different bacterial species have a common structure [3] as shown in Fig. 2. Endotoxin (LPS) consists of a polysaccharide and a covalently bound lipid termed lipid A. In LPS of wild type — (smooth or S-form) *Enterobacteria* the polysaccharide component consists of two regions which differ in the genetically determined biosynthesis and chemical structure: O-specific chains and a core oligosaccharide.

The 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.

The nature, sequence, type of linkages and substitution of the 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 different bacterial species is limited. Thus in *Salmonella* only one core type exists in all serotypes, and in *E. coli* so far six core types: Ra, R1, R2, R3, R4, and K12 have been described in more than a hundred different serotypes (Fig. 3).

The core region of enterobacterial lipopolysaccharides consists of a heterooligosaccharide which can be subdivided into the lipid A-proximal inner core and lipid A-distal outer core. The limited structural variability of the core types relates primarily to the outer region, while the Kdo-containing inner core appears to be more conserved [3]. The outer core contains the common sugars: glucose, galactose, glucosamine or galactosamine and is also called the hexose region. The inner core region is composed of the unusual sugars: heptose and 2-keto-3-deoxyoctulosonic acid (Kdo). These residues are usually substituted by charged groups such as phosphate, pyrophosphate, phosphorylethanolamine or pyrophosphorylethanolamine, often in nonstoichiometric amounts. Therefore the inner core region exhibits considerable accumulation of charged residues. The differentiation of core regions based on sugar composition is, however, not very precise.

The Enterobacterial Common Antigen (ECA) is present in a free cyclic [4] or glycolipid form in all enterobacterial strains (Fig. 4). This antigen was also found in a linked form in a few strains having R1 or R4 core type. Unfortunately, the nature of its linkage to the core OS is not known yet.

POLYSACCHARIDE
PHOSPHOLIPID
O-SPECIFIC CHAIN
OUTER CORE
INNER CORE
LIPID-A
P—EtN
P
P—EtN KDO
GlcNAc Gal Hep P KDO GlcN
D C B A [D C B A]n Glc—Gal—Glc—Hep—Hep—KDO—GlcN
P P
Ra Rb Rc Rd Re
VERY HIGH
MODERATE
LOW
NO
VERY LOW
STRUCTURAL VARIABILTY

Fig. 2. General structure of lipopolysaccharides (after [3]).

Chemotype **Structure**

R1 αD-Gal 1.2αD-Gal 1.2αD-Glc 1.3αD-Glc 1.3-inner core
3
|
1
βD-Glc

R2 αD-GlcNAc 1.2αD-Glc 1.2αD-Glc 1.3αD-Glc 1.3-inner core
6
| αD-Gal 1.7Kdo
1
αD-Gal

R3 αD-Glc 1.2αD-Glc 1.2αD-Gal 1.3αD-Glc 1.3LαD-Hep 1.3LαD-Hep 1.5 Kdo
3 7 4
| | |
1 1 2
αD-GlcNAc LαD-Hep Kdo 2.4Kdo
7
|
1
αD-GlcNAc

R4 αD-Gal 1.2αD-Gal 1.2αD-Glc 1.3αD-Glc 1.3-inner core
4
|
1
βD-Gal

K-12 LαD-Hep 1.6αD-Glc 1.2αD-Glc 1.3αD-Glc 1.3LαD-Hep 1.3LαD-Hep 1.5Kdo
6 7 4
| | |
1 1 2
αD-Gal LαD-Hep Kdo
|
Kdo 2.4 or αL-Rha 1.5

Fig. 3. Structures of the core oligosaccharides of E. coli R1-R4 (after [3]).

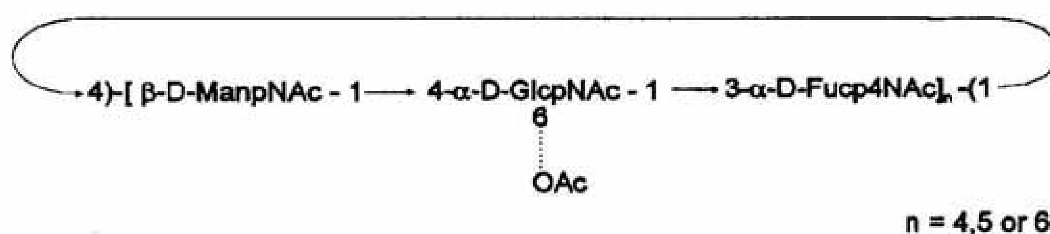

Fig. 4. The structure of cyclic form of Enterobacterial Common Antigen.

Lipid A is a covalently linked lipid component of LPS (Fig. 5). 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 β(1→6)glucosamine disaccharide and usually four moles of 3-hydroxymyristic acid. Two of them are amide linked and two form esters at position 3 and 3′. Both amide and ester bound hydroxy fatty acids are partially substituted by saturated fatty acids. Structural variability of lipid A is rather low [3].

Lipopolysaccharides exhibit heterogeneity which extends to all three regions: O-specific chains, core oligosaccharides and lipid A. Thus, enterobacterial S-form preparations contain a collection of lipopolysaccharide species which differ in the number of repeating units, i.e. in the length of the O-specific chain. The lipopolysaccharide fractions can be separated on the PAGE in the presence of SDS and visualised by silver staining or separated from the water soluble fraction after mild acid hydrolysis of LPS on a Bio-Gel P-10 column.

Endotoxins are responsible for initiation of septic shock, which increases the number of fatalities in Gram-negative bacteremia among hospital patients. The mortality from septic shock is still high despite recent developments in antibiotic therapy because antibiotics are unable to decrease the level of free lipopolysaccharides in the bloodstream. Moreover, it has been demonstrated that a significant amount of endotoxin can be released from killed bacterial cells following administration of antibiotics [5]. Prevention and treatment of septicemia could involve stimulation of an immune response against LPS. It was found that immunization with endotoxin core structures protected animals against bacterial infections and endotoxic shock. These antisera are of great interest because they are directed against conserved parts of LPS and therefore could have cross-reactive and cross-protective properties with respect to many Gram-negative rods.

We described earlier the possibility of obtaining a good non-toxic immunogen which did not contain lipid A, by conjugation of lipopolysaccharide core with tetanus toxoid [6]. Anticonjugate sera were bactericidal towards homologous serotypes of encapsulated *Neisseria meningitidis* B. Unfortunately, the conjugate contained a potentially carcinogenic aromatic amine unacceptable in a vaccine designed for humans.

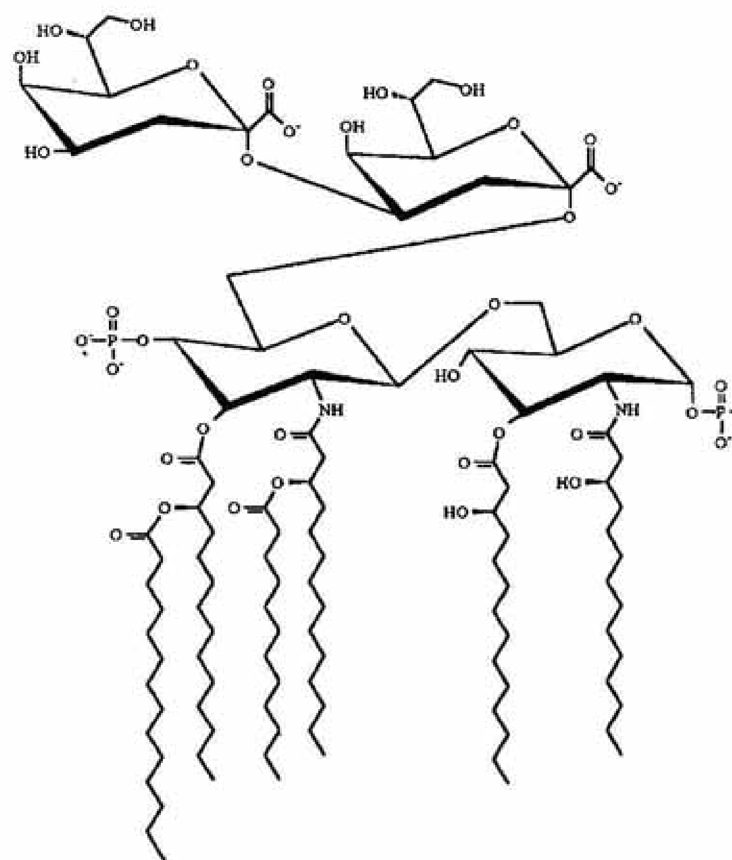

Fig. 5. The structure of Re type lipooligosaccharide.

Table 1
The level of antibodies in anti-conjugate sera

Serum	Antigen	Quantitative microprecipitin test		Immunoenzymatic test (ELISA)
		mg Ab/ ml		IgG
anti-OSPhI-TT (R1)	LPS	PhI	1.80	1.8×10^6
		PhII	1.60	1.5×10^6
		O14	0.70	6.0×10^5
anti-OSPhII-TT (R1)	LPS	PhI	2.10	1.7×10^6
		PhII	2.30	2.0×10^6
		O14	0.85	5.0×10^5
anti-OSO14-TT (R4)	LPS	PhI	0.60	4.0×10^5
		PhII	0.55	4.0×10^5
		O14	2.20	2.6×10^6
anti-ECA-TT	ECA		4.80	4.8×20^6

ECA, enterobacterial common antigen.

To prepare a new non-toxic, non-carcinogenic conjugate we chose the OS core of R1 type which is present in 70% of *E. coli* strains isolated from the blood of bacteremic patients.

The OS core was prepared after mild acid hydrolysis of *Shigella sonnei* LPS and linked covalently to the tetanus toxoid (TT) by reductive amination [7].

Controlled periodate oxidation did not destroy antigenic determinants of OS core. The antigenic activity of OS core conjugated to the protein was higher than of that native OS core, suggesting better exposure of the antigenic determinants on the surface of the protein carrier containing ten OS core molecules linked to one TT molecule. The conjugate was a good immunogen in rabbits yielding a high level of anti-OS core antibodies of IgG class (Table 1). This antibody was used to determine antigenic relationship between enterobacterial strains having different core types. The reaction of partial identity between R1 and R4 core types observed on rocket-line immunoelectrophoresis reflected the subtle differences between OS core of *Sh. sonnei* phase II and *E. coli* O14 [7]. The anti-OS core-TT serum was used at a concentration of 2 μg/ml to study its reaction with LPS isolated from several enterobacterial strains. This antiserum reacted with the fast migrating fraction of those LPS which were related to the unsubstituted core. Only the reaction between identical or homologous core types was observed. The anticonjugate serum reacted with intact, live, smooth bacterial cells. The linked antibody was labelled with fluorescein and the preparations obtained were examined in a fluorescein activated cells sorter. It was found that 85% of *Sh. sonnei* Ph I cells were labelled with the anti-OS core antibody in comparison to 93% labelled with the anti-O-specific chain antibody. When more concentrated anti-OS core-TT serum containing 20 μg Ab/ml was used in the immunoblotting test it was found that this antibody was able to react also with a high molecular mass fraction of LPS from smooth cells having core OS substituted by O-antigens. This suggested the presence in antiserum of a population of antibodies which were able to react with such LPS.

The anti-OS core-TT antisera used in protection studies showed high protective activity. At the concentration of 10-60 ng Ab/animal they protected mice completely against LD_{100} of smooth live bacteria of *Sh. sonnei* (4×10^7) and rough encapsulated *E. coli* O14 K7 (8×10^7) [8].

In conclusion the conjugates of OS core with tetanus toxoid are non-toxic, T-dependent antigens. The protein carrier in these glycoconjugates is provided by tetanus toxoid an universally approved as a vaccine in humans. The conjugates contain a reduced Schiff base which has been earlier found in animal tissue.

Complete core structures present in conjugates are also found in unsubstituted form in lipopolysaccharides of smooth cells and also on bacterial surface. These properties of OS core-TT conjugates suggest their potential use as a vaccine for humans against enterobacterial septicemia and endotoxic shock.

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