Immunobiological characterization of low toxic & pyrogenic lipopolysaccharide from *Salmonella typhi* P-4

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Abstrak

Lipopolisakharida (LPS) Salmonella typhi *adalah satu dari LPS kuman Enterobacteriaceae yang paling toksik dan pirogenik. Dengan menggunakan metode separasi dan purifikasi yang sesuai telah berhasil diisolasi LPS yang relatif kurang pirogenik dari* S. typhi *O:901. Pirogenitas LPS diperiksa dengan menggunakan kelinci sesuai dengan protokol standar WHO/Eu.Ph. untuk vaksin polisakharida. Dosis maksimum apirogenik (berat kering LPS per kg berat kelinci) – 0,025 µg. Pirogenitas yang rendah dari LPS dihubungkan dengan berkurangnya aktivitas* limulus gelating *(LAL assay) in vitro. Konsentrasi ambang yang menyebabkan tes LAL positif diperkirakan berada pada kisaran 0,004 - 0,0008 µg/ml. Ambang pirogenitas LPS tip tradisional dengan pirogenitas tinggi pada kelinci Westphal ditemukan secara bermakna lebih rendah, yaitu <0,00016 µg/ml. Injeksi LPS dosis 100, 150 mg/kg tidak menyebabkan kematian mencit (CBAxC57B1/6) F1. Mencit diinjeksi dengan LPS dosis 1, 5, 10, 25 µg, yang diperkirakan apirogenik dengan menggunakan koefisien pengenceran 1:2000 untuk uji vaksin polisakharida. Imunisasi mencit dengan LPS menginduksi respon imun primer. Antibodi spesifik O:9 LPS dideteksi di dalam serum mencit yang diimunisasi. Hasil yang diperoleh menunjukan aktivitas imunobiologik, seperti pirogenitas dari LPS, yang sesuai dengan parameter pirogenitas: kontrol 0.025 µg/kg dari WHO untuk vaksin polisakharida yang dimurnikan (tifoid, menigokokus). Pendekatan untuk kemungkinan aplikasi LPS dengan pirogenitas rendah sebagai imunogen protektif atau ajuvan masih dalam penelitian.*

Abstract

Lipopolysaccharide (LPS) Salmonella typhi *is one of the most toxic & pyrogenic among LPS's of Enterobacteriacae. Using combination of appropriate methods for separation and purification we were successful in isolation of relatively low pyrogenic lipopolysaccharide (LPS) from* S. typhi *O:901. Pyrogenicity of LPS was examined on rabbits according to standard WHO/Eu.Ph. protocol for polysaccharide vaccines. Maximal apyrogenic dose (dry weight of LPS per kilogram of rabbit weight) was 0.025 µg. Low pyrogenicity of LPS was correlated with decreased Limulus gelating activity (LAL assay) in vitro. Threshold concentrations which caused positive LAL test were estimated within a range 0.004 - 0.0008 µg/ml. The similar thresholds evaluated for traditional highly-pyrogenic for rabbits Westphal - type LPS were detected in significantly low concentration zone - below 0.00016 µg/ml. It was registered decreased endotoxicity for low-pyrogenic LPS samples. Injection of LPS in doses 100 and 150 mg/kg didn't cause the death of (CBAx C57Bl/6)F1 mice. Mice were injected with doses of LPS 1, 5, 10, 25 µg, which may be calculated as apyrogenic by use dilution coefficient 1: 2000 for testing polysaccharide vaccines. Primary immune response was induced after immunisation of mice with LPS. O:9 LPS-specific antibodies have been detected in sera of immunized mice. The results obtained indicate that such immunobiological activity as pyrogenicity of our LPS met WHO pyrogenicity control parameter 0.025 µg/kg applied for purified polysaccharide vaccines (typhoid, meningococcal). The approaches to possible application of low pyrogenic LPS as protective immunogen or adjuvant for vaccine construction is still under investigation.*

INTRODUCTION

Lipopolysaccharides (LPS's) represent one of the most attractive type of biologically active molecules from the vaccine construction immunomodulation viewpoint. LPS's manifest excellent protective properties[3] and may be used as a vaccine components but high toxicity and pyrogenicity interfere with such application.

During many years a lot of efforts have been undertaken to diminish the negative biological characteristics of the LPS's and at the same time maintaining their profitable properties.

Some years ago we developed original technology to obtain low-toxic and low-pyrogenic lipopolysaccharide (LTP-LPS) from *Salmonella typhi* without any chemical actions. In this paper, the summary of our studies of LTP-LPS as possible vaccine immunogen is presented.

PYROGENICITY

Pyrogenicity is essential for LPS immunobiological

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characteristic. Pyrogenicity experiments were carried out *in vivo* - on Rabbits, and *in vitro* by use of *Limulus* Àmebocyte Lysate (LAL) test.

In vivo

Pyrogenicity assay on rabbits have been done according to standard procedure (Eu.Ph.). Groups of rabbits were injected with different doses of LTP-LPS and LPS-Westphal type(LPS-W) for determination maximal threshold apyrogenic dose.

Threshold apyrogenic dose for LTP-LPS - 0.025 μg is greatly differ from this characteristic of LPS-W (Table 1).

More than 20-fold difference based on this data shall be marked. It should be noted that 0.025 μg per 1 kg of rabbit body weight is apyrogenic threshold approved by WHO, Eu.Ph. for meningitis or typhoid capsular polysaccharide vaccines.

Table 1. Pyrogenicity characteristics of the LTP-LPS and LPS-W in vivo

Sample	Threshold apyrogenic dose (μg per 1 kg rabbit body weight)	Dose of apyrogenic Immunisation (μg)
LTP-LPS	0.025	50
LPS-W	0.001	2

Dose of apyrogenic immunisation shall be calculated using dilution coefficient 1:2000 from pyrogenicity testing protocol for polysaccharide vaccines. This dose consist of 50 μg for LTP-LPS.

In vitro

Standard E-TOXATE® kit (Sigma, USA) was used for in vitro testing. LP-LPS showed a marked 100-fold decrease in *Limulus*-gelating activity in comparing with LPS-W (Table 2).

Chromatographically purified LPS-W *S.typhi* (Sigma, USA) has the same positive threshold parameters as non-chromatographied LPS.

Table 2. Pyrogenicity characteristics of the LTP-LPS and LPS-W in vitro

Sample	Positive LAL-test threshold dilution	Positive LAL-test threshold concentration μg/ml
LTP-LPS	1/50000	0.02
LPS-W	1/6250000	0.00016
LPS-W *S.typhi* Chromatographically purified Sigma L-2387	1/6250000	0.00016

ENDOTOXICITY

Acute toxicity LTP-LPS experiments were carried out on mouse model. LTP-LPS Lot's is well tolerated by mice in doses 2 or 3 mg.

Table 3. Endotoxicity characteristics of the LPT-LPS

Sample	Dose μg/mouse	Dose μg/kg	Survival of mice (CBAxC574B6) F1
LTP-LPS Lot#18	3000	150	10/10
LTP-LPS Lot#19	2000	100	10/10

Experimental data demonstrated safe immunisation of mice by LTP-LPS presented in the Table 3. Dose of safe immunisation with ordinary LPS-W is rarely exceed 500 μg per mouse. It should be noted that dose of safe immunisation by high-affinity complex LPS-myelin basic protein – 50-75 mg/kg[2]. Recently developed complex LPS-synthetic peptide was safely injected in mice in dose 60 mg/kg[1].

SEROLOGY

LPS properties as protective immunogen are closely connected with O-specific polysaccharide chains of LPS molecule[3]. O-Specific polysaccharide mainly determine protective immune specificity induced by LPS. Nativity, functional activity of O-antigenic determinants in LTP-LPS samples in comparison with LPS-W have been investigated by immunochemical methods.

Serological activity of LPS preparations was analyzed by passive haemagglutination reaction inhibition test. *S.typhi* LPS-W adsorbed erythrocytes and monospecific O:9 antiserum (Diagnostic Pasteur,

France) were used in concentration 4 SU. Serial dilutions of LPS from the concentration 100 μg/ml were added to microtitration wells.

LTP-LPS, LPS-W showed a similar inhibition activity (Table 4) as like as chromatographically purified LPS *S.typhi*, obtained from Sigma (USA).

ELISA-technique was used for the study of O:9-specific antigen-antibody interactions too.

Table 4. Inhibition point concentration in passive O:9-specific haemagglutination reaction of the LTP-LPS and LPS-W

Sample	Inhibition point concentration (μg/ml) of passive O:9-specific haemagglutination reaction
LTP-LPS	3.125
LPS-W	3.125
LPS-W *S.typhi* chromatographically purified Sigma L-2387	3.125

Microtitration ELISA plates were coated with 10 μg/ml LTP-LPS and LPS-W in carbonate buffer (50 mM, pH - 9,6).

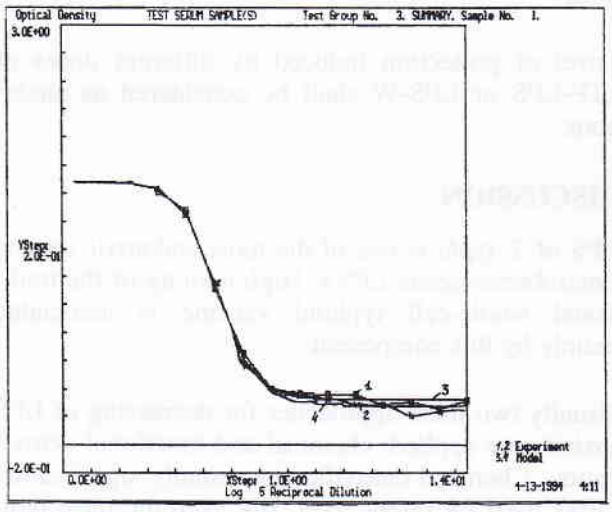

Figure 1. *Sample 1 - 2 LTP-LPS, Sample 3, 4, LPS-W*

Monospecific anti O:9 rabbit serum was added in serial dilutions. Peroxidase - labeled goat anti-rabbit Ig antibodies (Calbiochem, USA) were used as second antibodies.

The absorbance was measured at 492 nm in Dynatech ELISA reader, binding curves were analyzed by using Cyclop computer program.

Binding curves of O:9 antibodies by solid phase adsorbed LTP-LPS and LPS-W differ slightly (Figure 1). Serological data indicate, that O-antigenic characteristics of our LPS - sample are similar to traditional LPS-W and chromatographically - purified variant of LPS-W.

This provides evidence for non-alteration of O-antigenic polysaccharide sites during the process of obtaining LP-LPS.

IMMUNOGENICITY

Primary LPS-specific immune response was studied after immunization (CBAxC57Bl/6)F1 mice with LP-LPS and LPS-W.

IgG antibodies to LPS antigen were detected by ELISA. 96-Well plates were coated with 10 μg/ml LPS-W in carbonate buffer (50 mM, pH - 9,6). Peroxidase - labeled goat anti-mouse IgG antibodies (Calbiochem, USA) were used as second antibodies. End-point ELISA-titers were calculated by Cyclop computer program.

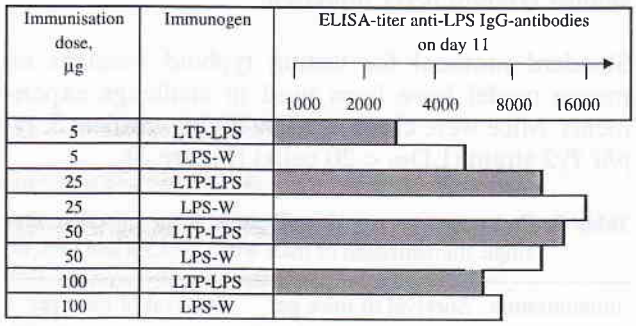

Figure 2. *Primary LPS-specific immune response after immunization with LTP-LPS and LPS-W*

All mice in groups immunized by LTP-LPS developed primary immune response registered on day 11 (Figure 2). High levels of antibodies were detected after immunisation with 25, 50 μg of LTP-LPS, whereas the animals receiving 100 μg showed a decrease LPS-specific response.

Comparison of serum samples after LTP-LPS and LPS-W immunization showed no, in most cases, sig-

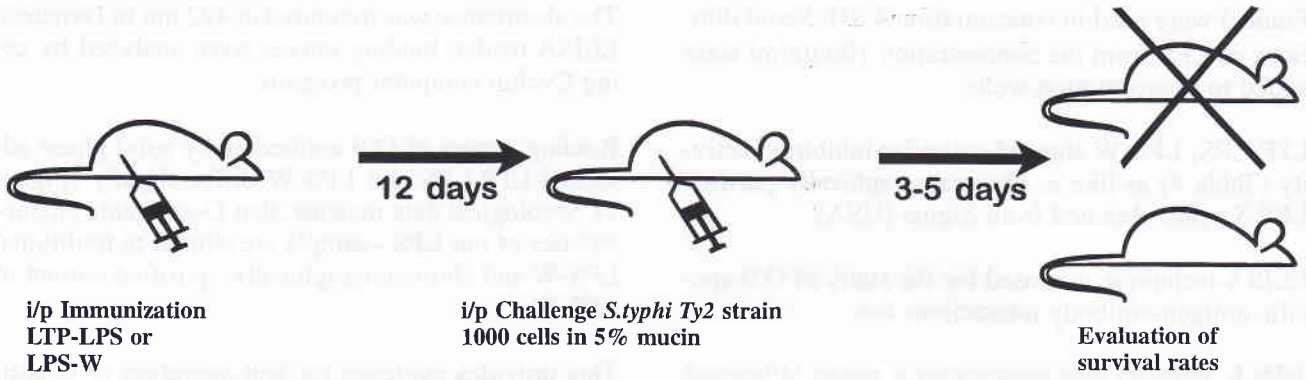

Figure 3. *Experimental protocols of the LTP-LPS and LPS-W protection in the challenge test*

nificant differences between levels of LPS-specific antibodies. Only mice injected with dose 5 μg of LTP-LPS elicited lower levels of specific antibodies than mice receiving the same dose LPS-W.

Dose-response relationship of primary immune responses induced by LTP-LPS and LPS-W are found to be in general agreement.

PROTECTIVE ACTIVITY

We investigated protective activity of LP-LPS against typhoid fever infection.

Standard protocol for testing typhoid vaccines on mouse model have been used in challenge experiments. Mice were challenged by highly-virulent *S. typhi Ty2* strain (LD_{50} < 20 cells) (Figure 3).

Table 5. Protection rates against Typhoid fever infection after single immunization of mice with LTP-LPS and LPS-W

Immunisation dose (μg)	Survival of mice per group immunised with LTP-LPS	Survival of mice per group immunized with LPS-W
25	10/10	8/10
5	8/10	7/10
1	5/10	6/10
0.2	8/10	6/10
0.04	6/10	4/10
0.008	5/10	6/10
0.0016	2/10	4/10
-	0/10	0/10

ED_{50} LTP-LPS - 0.037 μg;
ED_{50} - LPS-W - 0.081 μg.

Single LTP-LPS immunisation with dose 25 μg provided effective 100% protection against typhoid fever infection according to vaccine testing protocol (Table 5). This fact permits to evaluate LTP-LPS as possible protective immunogen under *S.typhi* infection. This dose 25 μg shall be evaluated as apyrogenic for LTP-LPS.

The low-dose immunization by using LTP-LPS and LPS-W (0.2; 0.04; 0.008; 0.0016 μg) protected 20 to 60 % of mice. This effect may be related with non-specific stimulation of immunity by these preparations.

Level of protection induced by different doses of LTP-LPS or LPS-W shall be considered as analogous.

DISCUSSION

LPS of *S. typhi* is one of the most endotoxic among Enterobacteriaceae LPS's. High toxicity of the traditional whole-cell typhoid vaccine is associated mainly by this component .

Usually two main approaches for decreasing of LPS toxicity are applied: chemical and functional detoxication. Chemical detoxification usually significantly raises the LPS safety level. But even the most delicate destruction of the native structure of lipid A portion may lead to negative modification of LPS immunogenic potential[4].

LPS functional detoxification is achieved by high-affinity binding of lipid A moiety by cationic peptide molecules, polymyxin B, myelin basic protein[2]. Recently detoxification of LPS have been performed by

binding with specially synthetized peptides[1]. Safe injection LPS complexed with peptides to mice have been registered in the increased dose range (50-75 mg/kg) compared with pure endotoxin.

The result of our investigation is a new variant of protective immunogen LTP-LPS with higher level of safety compare with classical LPS (Westphal - type). This higher level of safety is determined by low-pyrogenicity and low-toxicity.

LTP-LPS prepation have been obtained without using of foreign binding molecule or treating with any chemical agents. Successful injection of LPT-LPS to mice in doses 100, 150 mg/kg shown the higher level of safety for this immunogen than for functionally-detoxified LPS's. Pyrogenicity is one of the most serious negative characteristic of vaccines. It connects with many side reactions and gives a lot of troubles to the developers and producers of vaccines. LTP-LPS fully meet WHO pyrogenicity requirements to polysaccharide vaccines.

It should be stressed that LTP-LPS showed the same level of immunogenic and protective properties as traditional LPS-Westphal. LTP-LPS induced O-specific humoral immune response, acquired immunity against typhoid fever, increased mean survival time for mice Balb/c inoculated by mastocytoma P815.

Attempts of vaccine construction using LPS as a main and additional protective immunogen have been permanently undertaken. Using of LTP-LPS is one of the possible way of this problem decision.

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