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# Investigation of Influence of *Proteus mirabilis* LPS Polysaccharide Part on the Murine Immune Cells Activation

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**Abstract.** The biological activities were investigated of *Proteus mirabilis* lipopolysaccharides (LPSs) and their fragments, namely, the induction of nitric oxide (NO) and interleukin 1 (IL-1) synthesis by murine macrophages and the proliferation of murine spleen cells. The O-specific polysaccharide (F1), core oligosaccharide (F2) and lipid A were as effective as intact LPS in stimulating murine macrophages to produce NO. IL-1 synthesis was also induced by all studied types of endotoxins (S, Ra, Re) and partial structures, however F1, F2 and lipid A fractions required the presence of serum. In contrast to LPS, the O-specific polysaccharide, core oligosaccharide and lipid A were not able to induce the blast response of murine non-adherent splenocytes.

**Key words:** *Proteus mirabilis*; lipopolysaccharide; nitric oxide; interleukin 1.

## Introduction

The lipopolysaccharide (endotoxin) is the major component of Gram-negative bacterial outer membrane. It is composed of two chemically different parts. The hydrophilic polysaccharide domain is covalently bound via 2-keto-3-deoxyoctonic acid to hydrophobic lipid A. The polysaccharide (PS) fragment is subdivided into the inner and outer core oligosaccharide region and an O-specific chain (OPS) consisting of repeating units. The structural diversity is characteristic for polysaccharide fraction, while the lipid A is the most conservative part among different endotoxins[25].

LPS is implicated in the clinical syndrome of Gram-negative sepsis. The hypotension and multi-organ failure are the main symptoms of septic shock, and frequently lead to death. LPS is not toxic by itself, but exerts its effect via activation of the immunocompetent cells: monocytes, macrophages and neutrophils particularly. These cells are thought to play a central role in pathophysiology of Gram-negative septicemia through production of numerous biologically active mediators, including: free radicals (superoxide, hydrogen peroxide, hydroxyl radical, nitric oxide); proinflammatory cytokines (interleukin 1, interleukin 6, tumor necrosis factor) and prostaglandins[18, 22, 25].

The lipid A portion of lipopolysaccharide of Gram-negative bacteria is considered as the biological active center of whole heteropolymer. However the polysaccharide fragment can modulate the activity of LPS. The O-polysaccharide is known to activate B cells to induce the production of specific antibodies. It was also described that complement activation might be due to core oligosaccharide and O-polysaccharide parts of endotoxin[13]. Moreover, the isolated core part of *Salmonella minnesota* LPS stimulated the IL-1 secretion by human monocytes[17].

The aim of our studies was to investigate the biological activity of *Proteus mirabilis* LPS. We have prepared series of *P. mirabilis* endotoxins differing in PS

chain length isolated from: wild S1959 strains as well as from its rough mutants: R110/1959 (Ra) and R45/1959 (Re). Moreover we have examined purified S1959 LPS partial structures: core oligosaccharide (F2), O-specific polysaccharide (F1) and lipid A fragments.

## Materials and Methods

*The endotoxins*. The LPSs from smooth strain of *P. mirabilis* S1959 and its rough mutants R110 (Ra) and R45 (Re) types were used. S1959 LPS has not defective polysaccharide and lipid A domains. Both rough mutants lack of O-specific polysaccharide. R110 LPS contains the complete core oligosaccharide, while in R45 LPS core is reduced to two residues of 2-keto-3-deoxyoctonic acid (Kdo) and one of 4-aminoarabinose (Ara4N). The smooth LPS was extracted by phenol-water method[27], whereas rough forms by phenol-chloroform-petroleum ether procedure[9]. Crude S1959 LPS was purified by treatment with RNase and DNase, followed by ultracentrifugation at 100 000×g for 3 h[14]. All lipopolysaccharide preparations were electrodialyzed and transformed in sodium salt form. To prepare partial structures, S1959 LPS was cleaved by hydrolysis with 1% acetic acid (100°C, 2h). The precipitated lipid A was separated by centrifugation and washed with water. A water-soluble polysaccharide product was fractionated by gel chromatography on Sephadex G50 fine with 0.02 M pyridine/acetate buffer (pH 5.6) as the eluent, to obtain an O-specific polysaccharide (F1) and a core oligosaccharide (F2) fractions[23]. The *E. coli* O127: B8 LPS was purchased from Sigma (USA). All LPSs and partial structures at dose 1 mg/ml were prepared in PBS, and homogenous suspension was obtained by mixing the LPS solution in a sonication bath for 10 min. Finally, the solution was diluted in culture medium to the concentration used in the test system.

*Chemical reagents*. The mouse interferon γ (IFN-γ) was the kind gift of Dr. In-Pyo Choi (KRIBB, Republic of Korea). Sulfanilamide, naphthylethylenediamine dihydrochloride, phosphoric acid, MTT (3-[4,5-dimethylthiazol-2-yl]-2.5 diphenyl tetrazolium bromide), concanavalin A (ConA), sodium m-periodate, formaldehyde, ammonium persulfate, TEMED, β-mercaptoethanol, were purchased from Sigma (USA). Tris, citric acid, glycine, dodecylsulfate were obtained from Serva (Germany). Silver nitrate, acetic acid, $NH_4OH$, NaOH were from POCh (Poland). Thirty percent acrylamide/bis was purchased from Bio-Rad (USA). Chloroform, methanol, ethylacetate, ether were obtained from Merck (Germany). HCl was purchased from Aldrich (Germany).

*Cells*. The RAW 264.7 macrophage-like cell line was cultured in DMEM medium (Sigma) supplemented with 10% FBS (Cansera, Canada), 100 U/ml of penicillin and 100 μg/ml of streptomycin (Gibco-BRL) at 37°C with 5% $CO_2$.

The murine peritoneal macrophages were obtained from C57BL/6 female mice intraperitoneally injected 3 days before with 1 ml 10% proteose peptone. The cells were collected by peritoneal washing with ice-cold PBS with 2% FBS and 0.117 mg/ml EDTA. The peritoneal cells contained 70% of macrophages, which was determined by staining with Giemsa dye solution.

*Gass chromatography/mass spectrometry*. The analysis of fatty acid content in specimens was performed according to WOLLENWEBER and RIETSCHEL[28]. Briefly, fatty acids were liberated by acid hydrolysis of LPS (4 N HCl, 4 h, 100°C) and extracted from the reagent mixture with chloroform (3 times). Estrification was performed 6 h at 85°C with 2 N HCl solution in methanol. After cooling, the mixture was concentrated to half of initial volume with a stream of nitrogen and supplemented (1:1) with half-saturated aqueous NaCl. Next, the extraction of fatty acid methyl esters was performed with the 1:1 (v/v) mixture of ethyl acetate and ethyl ether. The final product was analyzed by gass chromatography/mass spectrometry (GC/MS) with model 6890A instrument (Hewlett-Packard) equipped with HP-5MS capillary column (30 m by 0.2 mm), autosampler HP 6890 series injector and mass spectrum detector HP 5973 MSD with electron impact (EI) employing an ionization potential of 70 eV. The column temperature was computer-programmed to increase from 70 to 280°C at the rate of 8°/min.

*SDS-PAGE with silver-staining*. LPS S1959 (20, 10, 1 μg); lipid A (10, 1, 0.1 μg); F1 (100, 20, 10 μg) and F2 (100, 20, 10 μg) were subjected to SDS-PAGE, using 3.5% stacking and 12.5% separating gel, in the LAEMMLI[16] system. After electrophoresis, the gel was stained with silver nitrate according to TSAI and FRASCH[24].

*Interleukin 1 assay*. Quantitation of IL-1 produced by RAW 264.7 cells was performed by measuring the supportive activity for thymocytes[11]. Briefly, RAW 264.7 cells ($2 \times 10^5$ cells/well in 96 well plate) were incubated for 4 h at 37°C with 5% $CO_2$ in complete DMEM medium. After removing the medium, the cells were washed twice with serum-free DMEM. The endotoxins at concentrations $10^{-6}$–10 μg/ml in DMEM without or with 10% FBS were added to final volume of 200 μl/well. In some plates a medium without LPS was added as a control. The plates were incubated further for 24 h at 37°C with 5% $CO_2$.

The thymocytes suspension from BALB/c female mice were distributed at a concentration of $4 \times 10^5$ cells/well in 96 well plate in RPMI 1640 medium (Sigma) supplemented with 10% FBS in 100 µl final volume. The 100 µl of culture supernatants for the RAW 264.7 treated with LPSs or control were added to the thymocytes culture and the plates were incubated for 72 h at 37°C with 5% $CO_2$. The proliferation of thymocytes was estimated with MTT assay[1].

Because of the possibility that culture supernatants transferred for IL-1 assay might still contain endotoxins, we tested whether LPS itself had the ability to induce proliferation of thymocytes. The *P. mirabilis* as well as *E. coli* LPS alone did not stimulate blast response of thymocytes, whereas ConA activated them to proliferation by itself under the same conditions.

*Measurement of nitric oxide.* The production of nitric oxide (NO) was measured in culture supernatant by quantitating the nitrite ($NO_2^-$), a stable metabolite of NO, using Griess reagent (1% sulfanilamide mixed with 0.1% naphthylethylenediamine dihydrochloride in 5% phosphoric acid)[10]. The RAW 264.7 cells or freshly isolated peritoneal macrophages (PM) were distributed in 96 well plate ($2 \times 10^5$ cells/well) in DMEM with 10% FBS. After allowing cells to attach to plastic culture dishes for 4 h at 37°C with 5% $CO_2$ in complete DMEM medium, the non-adherent cells were removed. The adherent cells were washed twice with PBS and treated with LPSs ($10^{-1}$ µg/ml) or INF-γ (10 or 100 U/ml) or LPSs ($10^{-1}$ µg/ml) and IFN-γ (10 or 100 U/ml) simultaneously, dissolved in PBS with 5% FBS. After incubation for 24 h, 100 µl of culture supernatants were transferred into microtiter plate and reacted with 100 µl of Griess reagent for 10 min at room temperature. The absorbance was measured at 540 nm by microplate reader (Bio-Rad, USA). The amount of nitrite was calculated from $NaNO_2$ standard curve.

*Blast response of mouse spleen cells.* The spleen cells were obtained from C57BL/6 female mice. The erythrocytes were lysed in distilled water. The adherent cells were removed by whole spleen cells adherence to plastic Petri dishes for 2 h. The non-adherent splenocytes ($4 \times 10^5$ cells/well in 96 well plate) were incubated for 72 h with LPS (1 µg/ml) or ConA (2.5 µg/ml) in RPMI 1640 medium supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C with 5% $CO_2$. The proliferation of spleen cells was measured using MTT assay. This method is faster than the $^3$H-TdR uptake assay, allows to avoid the radioactive material and is sufficient for study the growth and survival of mammalian cells[1].

*Statistics.* Results are given as a means ± standard deviation (SD). Statistical analysis was performed using Student's *t*-test. Statistical significance was defined as $p \leq 0.05$.

## Results

### *The analysis of lipid A contamination in saccharide partial structures*

One of the specific methods for determination of lipid A contamination in F1 and F2 fractions is the 3-hydroxytetradecanoic acid (3-OH 14:0) methyl ester analysis with the help of GC/MS system. The 3-OH 14:0 is known to be most characteristic fatty acid present in enterobacterial lipid A. Data presented in Table 1 and Fig. 1 show that in both polysaccharide (F1) and

**Table 1.** The fatty acids content in lipopolysaccharide and its saccharide partial structures

| | F1 (µg/mg) | F2 (µg/mg) | LPS S1959 (µg/mg) |
|---|---|---|---|
| 14:00 | nd | nd | 172.6 |
| 3-OH 14:0 | nd | nd | 184.4 |
| 16:00 | 3.1 | 2.7 | 64.4 |

The amounts of fatty acid were analysed in relation to heptadecanoic acid (as an internal standard) with the ratio of 15 µg of heptadecanoic acid to 1 mg of LPS and 1.5 µg to 1 mg of F1 or F2 fractions. For analysis about 2 mg of each specimen (LPS, F1, F2) were used.

nd – not detected.

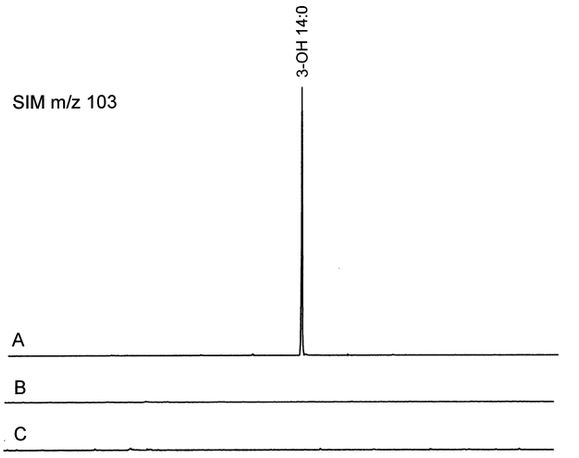


**Fig. 1.** GC analysis of 3-hydroxytetradecanoic acid content in LPS S1959 (A) and its saccharide fractions: F2 (B) and F1 (C). The amount of 3-OH 14:0 was detected in relation to heptadecanoic acid (as an internal standard) with the ratio of 15 µg of heptadecanoic acid to 1 mg of LPS and 1.5 µg to 1 mg of F1 or F2 fractions. For analysis about 2 mg of each specimen (LPS, F1, F2) were used. Data presents the SIM (selected ion monitoring) of ion m/z 103 which is the most characteristic for acid being investigated

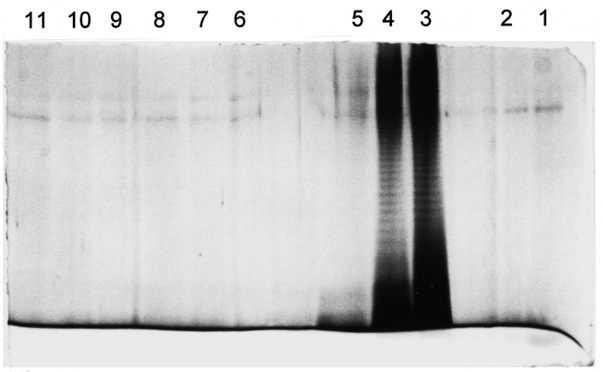


**Fig. 2.** Silver-stained SDS-PAGE banding pattern of. *P. mirabilis* S1959 LPS and their partial structures. Lines: 1 – lipid A 10 μg; 2 – lipid A 1 μg; 3 – S1959 20 μg; 4 – S1959 10 μg; 5 – S1959 1 μg; 6 – F1 100 μg; 7 – F1 20 μg; 8 – F1 10 μg; 9 – F2 100 μg; 10 – F2 20 μg; 11 – F2 10 μg

oligosaccharide (F2) fractions no 3-hydroxytetradecanoic acid was detected. This suggested that saccharide partial structures were free of lipid A.

Similar results were obtained when SDS-PAGE, silver-staining method was employed (Fig. 2). In contrast to the lipid A preparation (lanes 1, 2), no characteristic band was observed neither in F1 (lanes 6–8), nor in F2 (lanes 9–11), independently of dose used. These data were confirmed with the help of thin layer chromatography (TLC) also (data not shown).

### *IL-1 production by RAW 264.7 cells stimulated with P. mirabilis lipopolysaccharides and their partial structures*

In the preliminary investigations the effect of various endotoxin doses on IL-1 production by RAW 264.7 cells in serum free medium was studied. The results presented in Fig. 3 might suggest that all LPS chemotypes studied (S, Ra, Re), as well as LPS partial structures (F1, F2, lipid A) showed dose dependent ability to induce IL-1 synthesis. In the further experiments we have decided to use the dose of LPS from a middle of plateau $10^{-1}$ μg/ml.

We compared the level of IL-1 production by RAW 264.7 cells stimulated with LPSs in serum free condition and in medium supplemented with 10% FBS. As it is shown in Fig. 4, in serum free medium the significant IL-1 production was observed when cells were treated with S1959, R110 (Ra) or R45 (Re) LPSs only. However purified F1, F2 and lipid A fractions did not induce IL-1 releasing in such conditions, which revised our preliminary supposition. Contrary, in the presence of bovine serum, all three fractions were able to induce IL-1 production on similar to S1959 LPS level. It indicated that certain serum factors facilitate the effect of endotoxin on IL-1 production.

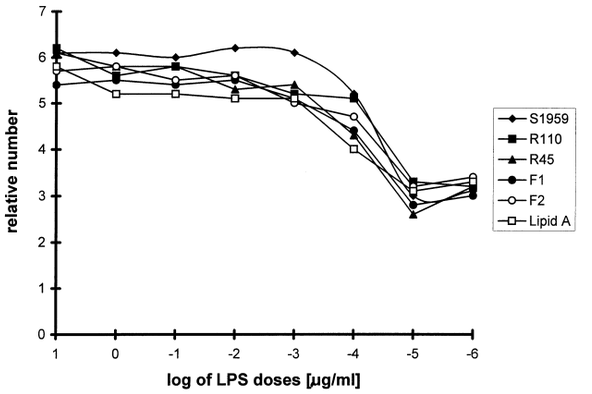


**Fig. 3.** The dose dependent effect of *P. mirabilis* lipopolysaccharides and their partial structures on IL-1 production by RAW 264.7 cells. The RAW 264.7 cells were incubated 24 h with LPSs $10^{-6}$-10 μg/ml in DMEM medium. IL-1 activity in culture supernatants was tested in primary culture of thymocytes. Data are from one representative experiments. The spontaneous proliferation of non-stimulated thymocytes (OD=0.116) was evaluated as relative number 1

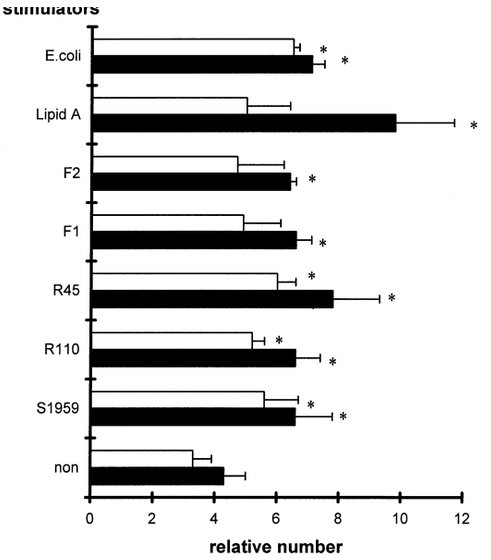


**Fig. 4.** IL-1 production by RAW 264.7 cells after stimulation with *P. mirabilis* lipopolysaccharides and their partial structures. The RAW 264.7 cells were incubated 24 h with LPSs ($10^{-1}$ μg/ml) in DMEM with 10% FBS (filled bar) or without serum (open bar). IL-1 activity in culture supernatants was tested in primary culture of thymocytes. Data are mean from 3 independent experiments ± SD. Statistical significance: LPSs vs. control * $p \leq 0.05$. The proliferation of non-stimulated thymocytes (OD=0.116) was evaluated as relative number 1

*The synthesis of nitrix oxide by murine macrophages stimulated with P. mirabilis lipopolysaccharides and their partial structures*

The capacity of smooth and rough LPS forms as well as LPS partial structures to induce NO synthesis by freshly isolated murine peritoneal macrophages (PMs) was investigated. As it is shown in Fig. 5, all tested bacterial products (saccharide F1 and F2 fractions including) exerted significant effect when PMs were co-stiumulated with IFN-γ and no effect was observed when used themselves.

Similar tendency was observed when RAW 264.7 cells were studied (Fig. 6), however only in the case of S1959 LPS (consisting of all three domains: lipid A, core oligosaccharide and O-specific polysaccharide) the stimulation was statistically significant.

*The blast response of non-adherent murine splenocytes to P. mirabilis lipopolysaccharides and their partial structures*

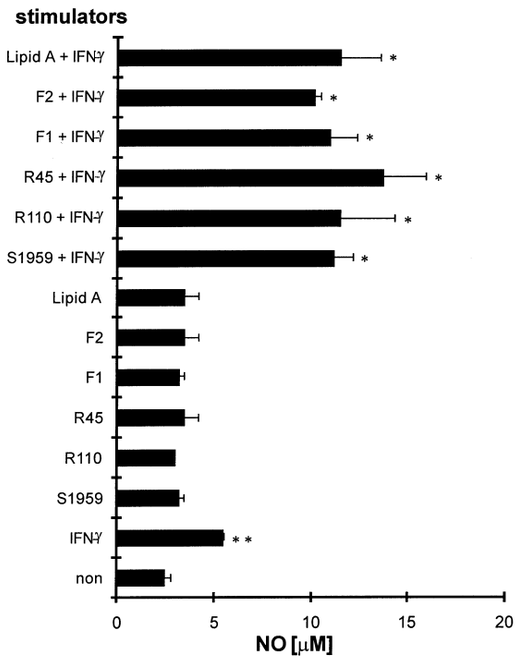


**Fig. 5.** NO formation by mouse peritoneal macrophages after stimulation with *P. mirabilis* lipopolysaccharides and their partial structures. Peritoneal macrophages were obtained from C57BL/6 mice intraperitoneally injected 3 days before with 1 ml 10% proteose pepton. Then cells were kept with LPSs ($10^{-1}$ μg/ml) or with IFN-γ (100 U/ml) or with LPSs ($10^{-1}$ μg/ml) and IFN-γ (100 U/ml) simultaneously for 24 h in PBS+5% FBS. Presented data are mean from 3 independent experiments ± SD. Statistical significance: LPSs+IFN-γ vs. IFN-γ * p≤0.05, IFN-γ vs. control ** p≤ 0.5.

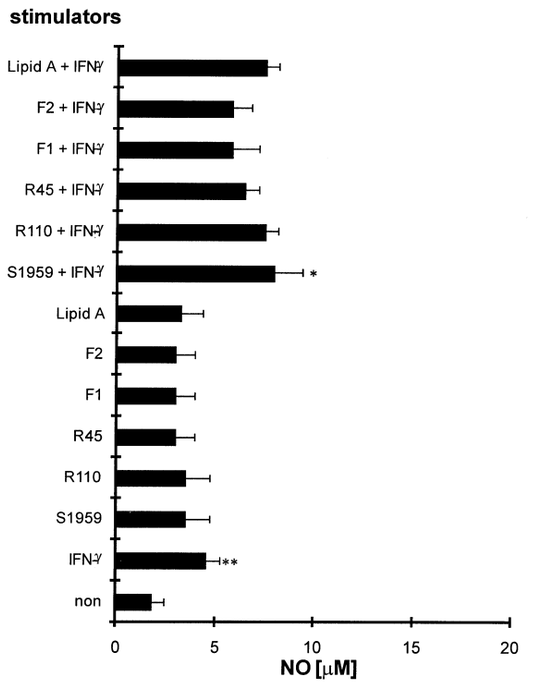


**Fig. 6.** NO formation by RAW 264.7 cells after stimulation with *P. mirabilis* lipopolysaccharides and their partial structures. The RAW 264.7 cells were incubated with: LPSs ($10^{-1}$ μg/ml) or IFN-γ (10 U/ml) or LPSs ($10^{-1}$ μg/ml) +IFN-γ (10 U/ml) simultaneously for 24 h in PBS supplemented with 5% FBS. Presented data are mean from 3 independent experiments ± SD. Statistical significance: S1959+IFN-γ vs. IFN-γ * p≤0.05, IFN-γ vs. control ** p≤0.05

The ability of a series of differing in saccharide chain lenght endotoxins to induce the blast response of splenocytes was investigated. As it is shown in Table 2, LPS of all chemotypes activated murine non-adherent spleen cells in similar degree. Contrary, the O-specific polysaccharide and the core oligosaccharide fragments failed to stimulate a proliferation of non-adherent spleen cells.

## Discussion

The lipid A was reported to be an endotoxin principle of lipopolysaccharide. Its key role in activation of macrophages is well known, while the significance of the polysaccharide part of LPS has not been profoundly investigated. We found that purified (no lipid A and non-hydrolyzed LPS – Fig. 1, Fig. 2 and Table 1) O-specific polysaccharide and core oligosaccharide fractions of *P. mirabilis* LPS were able to stimulate murine macrophages.

The production of pro-inflammatory cytokines and NO is a crucial step in inflammation. IL-1 is known to

**Table 2.** Blast response of non-adherent splenocytes stimulated with *P. mirabilis* endotoxins

| Stimulators of non-adherent spleen cells | Mean OD ± SD |
|---|---|
| Non | 0.392 ± 0.130 |
| ConA | 0.902 ± 0.207* |
| S1959 | 0.630 ± 0.040* |
| R110 | 0.623 ± 0.064* |
| R45 | 0.706 ± 0.050* |
| F1 | 0.405 ± 0.110 |
| F2 | 0.424 ± 0.116 |
| Lipid A | 0.620 ± 0.139 |

The non-adherent splenocytes were stimulated with LPSs (1 μg/ml) or ConA (positive control) for 72 h in complete culture medium or incubated in medium without any stimulators (negative control). Presented data are mean from 3 independent experiments ± SD. Statistical significance: LPSs vs. negative control * $p \leq 0.05$.

induce the fever, hypotension and acute phase protein[3, 8]. We showed that all LPS chemotypes studied: S, Ra and Re induced the IL-1 release by RAW 264.7 cells in the presence of 10% as well as without FBS (Fig 4), while for activation by isolated lipid A, F1 and F2 fractions the addition of serum was necessary. Many reports have documented the interaction of bacterial lipopolysaccharide with plasma proteins: lipopolysaccharide binding protein (LBP), albumin, transferin, $\alpha_2$ macroglobulin, complement. These factors are involved in expressing of the biological activity of endotoxin[5, 7]. It was reported that serum could selectively enhance NO synthesis and block IL-6 production by peritoneal macrophages stimulated with *E. coli* LPS[12]. However, it has not been a strong evidence that certain serum factors could mediate cell activation by polysaccharide part of LPS till now, such possibility cannot be postponed. It is also imaginable that the acivation pathway by PS is different from this by lipid A.

The nitric oxide plays an important role in the killing of foreign organisms, however its over-production may cause a tissue damage[2, 4, 19]. The LPS is known to be one of the NO production stimulators[6, 26]. A strong co-stimulatory effect of IFN-γ was observed in macrophages[2, 26]. In our experiments, the various, differing in PS chain lenght forms of *P. mirabilis* lipopolysaccharide, stimulated PMs as well as RAW 264.7 cells to release NO at similar levels (Fig. 5, Fig. 6). As expected, the simultaneous treatment with IFN-γ was the pre-requisite. Surprisingly, the purified core oligosaccharide and O-specific polysaccharide fragments were as potent as lipid A and intact LPS in inducing of NO production in such conditions. These results are to a certain degree parallel to demonstrated by KELLY et al.[15] who observed no close relationship between the amount of polysaccharide fraction in *Salmonella minnesota* LPS and the amount of TNF-α induced into RAW 264.7 cells supernatant. Moreover, OTTERLEI et al.[20] demonstrated that O-specific polysaccharide being D-ManA homopolymer was as efficient as whole LPS in induction of TNF-α synthesis by human monocytes cultured in the presence of serum. This may suggest lipid A not to be the only LPS part potent to stimulate monocytes/macrophages to produce and release inflammation mediators.

O-specific polysaccharide, core oligosaccharide and lipid A purified fractions, however able to stimulate macrophages for NO and cytokine production, failed in stimulation of non-adherent splenocytes to proliferation. It may be concluded that the activation of spleen cells by LPS fragments does not have to be accompanied by proliferation, or such products are not able to activate them at all in conditions applied. In supporting the first hypothesis, the mannose homopolymer as a O-specific polysaccharide had strong adjuvant and anticomplementary activity, however did not activate murine B cells to proliferation[21]. It should be stressed that receptors for various structural fragments of LPS await for extensive study, with the characterization of serum factors modulating the response.

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## References

1. BONOUS D. I., CAMPAGNOLI R. P. and BROWN J. (1992): Comparison of MTT calorimetric assay and tritiated thymidyne uptake for lymphocyte proliferation assay using chicken splenocytes. Avian Dis., **36**, 1022–1027.
2. BREDT D. S. and SNYDER S. H. (1994): Nitric oxide: a physiological messenger molecule. Annu. Rev. Biochem., **63**, 175–195.
3. CHUJOR C. S. N., KLEIN L. and LAM C. (1996): Selective inhibition of interleukin 1 gene expression in activated RAW 264.7 macrophages by interferon-γ. Eur. J. Immunol., **26**, 1256–1259.
4. DARLEY-USMAR V., WISEMAN H. and HALLIWELL B. (1995): Nitric oxide and oxygen radicals: a question of balance. FEBS Lett., **369**, 131–135.
5. DAVID S. A., BALARAM P. and MATHAN V. I. (1995): Characterization of the interaction of lipid A and lipopolysaccharide with human serum albumin: implications for an endotoxin carrier function for albumin. J. Endotox. Res., **2**, 99–106.
6. EIGLER A., MOELLER J. and ENDREAS S. (1995): Exogenous and endogenous nitric oxide attenuates tumor necrosis factor synthesis in murine macrophages cell line RAW 264.7. J. Immunol., **154**, 4048–4054.

7. FALK M. C., FLETCHER M. A., WILLIAMS T. J., CHING W. M., WU Y. and MORRISON T. K. (1996): Aggregation of serum proteins with lipopolysaccharide (LPS): characterization of the precipitable LPS-protein complex. J. Endotox. Res., **3**, 129–142.
8. FANTUZZI G., ZHENG H., FAGGIONI R., BENIGNI F., GHEZZI P., SIPE J. D., SHAW A. R. and DINARELLO C. A. (1996): Effect of endotoxin in IL-1β-deficient mice. J. Immunol., **157**, 291–296.
9. GALANOS C., LIIDERITZ O. and WESTPHAL O. (1969): A new method for the extraction of R-lipopolysaccharides. Eur. J. Biochem., **9**, 245–249.
10. GREEN L. C., WAGNER D. A., GLOGOWSKI J., SKIPPER P. L., WISHNOK J. S. and TANNENBAUM S. R. (1982): Analysis of nitrate, nitrite, and [$^{15}$N] nitrate in biological fluids. Anal. Biochem., **126**, 131–138.
11. HAMBLIN A. S. and O'GARRA A. (1987): Assay for interleukins and related factors. In KLAUS G. G. B. (ed.): Lymphocytes a practical approach. IRL Press, Oxford, 209–228.
12. HIROHASI N., RICHARDS M. and MORRISON D. C. (1996): Selective effects of serum on bacterial LPS-induced IL-6 and nitric oxide production in murine peritoneal macrophages. J. Endotox. Res., **3**, 395–405.
13. JOINER K., JIMENEZ-LUCHO V., GROSSMAN N., FOULDS J., FRANK M. and LEIVE L. L. (1988): *Salmonella* and complement: the critical influence of O-polysaccharide within LPS. In CABELLO F. C. and PRUZZO C. (eds.): Bacteria, complement and the phagocytic cells. Springer-Verlag, Berlin–Heidelberg, 139–155.
14. KACA W., KNIREL Y. A., VINOGRADOV E. V. and KOTEŁKO K. (1987): Structure of the O-specific polysaccharide of *P. mirabilis* S1959. Arch. Immunol. Ther. Exp., **35**, 431–437.
15. KELLY N. M., YOUNG I. and CROSS A. S. (1991): Differential induction of tumor necrosis factor by bacteria expressing rough and smooth lipopolysaccharide phenotypes. Infect. Immun., **59**, 4491–4496.
16. LAEMMLI U. K. (1970): Cleavage of structural proteins during the assembly of the head of bacteriophages T4. Nature, **227**, 680–685.
17. LEBBAR S., CAVAILLON J. M., CAROFF M., LEDUR A., BRADE H., SARFATI R. and HAEFFNER-CAVAILLON N. (1986): Molecular requirement for interleukin 1 induction by lipopolysaccharide-stimulated human monocytes: involvement of the heptosyl-2-keto-3-deoxyoctulosonate region. Eur. J. Immunol., **16**, 87–91.
18. LYNN W. A. and GOLENBOCK D. T. (1992): Lipopolysaccharide antagonists. Immunol. Today, **13**, 271–276.
19. NUSSLER A. K. and BILLIAR T. R. (1993): Inflammation, immunoregulation, and inducible nitric oxide synthase. J. Leukoc. Biol., **54**, 171–176.
20. OTTERLEI M., SUNDAN A., SKJAK-BRAEK G., RYAN L., SMIDSROD O. and ESPEVIK T. (1993): Similar mechanisms of action of defined polysaccharides and lipopolysaccharides: characterization of binding and tumor necrosis factor alpha induction. Infect. Immun., **61**, 1917–1925.
21. PAENG N., KIDO N., SCHMIDT G., SUGIYAMA Y. K., KOIDE N. and YOKOCHI T. (1996): Augmented immunological activities of recombinant lipopolysaccharide possessing the mannose homopolymer as the O-specific polysaccharide. Infect. Immun., **64**, 305–309.
22. RABINOVICI R., NEVILLE L. F. and FEUERSTEIN G. (1995): Current understanding of sepsis: criticism and a proposal. J. Endotox. Res., **2**, 163–168.
23. SIDORCZYK Z., SWIERZKO A., KNIREL Y. A., VINOGRADOV E. V., CHERNYAK A. Y., KONONOV L. O., CEDZYNSKI M., ROZALSKI A. and KACA W. (1995): Structure of epitope specificity of the O-specific polysaccharide of *Proteus penneri* strain 12 ATCC 33519 containing the amide of D-galacturonic acid with L-threonine. Eur. J. Biochem., **230**, 713–721.
24. TSAI C.-M. and FRASCH C. E. (1982): A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. Anal. Biochem., **119**, 115–119.
25. ULEVITCH R. J. and TOBIAS P. S. (1995): Receptor-dependent mechanisms of cell stimulation by bacterial endotoxin. Annu. Rev. Immunol., **13**, 437–457.
26. WEINBERG J. B., MISUKONOS M. A., SHAMI P. J., MASON S. N., SAULS D. L., DITTMAN W. A., WOOD E. R., SMITH G. K., MCDONALD B., BACHAUS K. E., HANEY A. F. and GRANGER D. L. (1995): Human mononuclear phagocytes inducible nitric oxide synthase iNOS.: analysis of iNOS mRNA, iNOS protein, biopterin and nitric oxide production by monocytes and peritoneal macrophages. Blood, **86**, 1184–1195.
27. WESTPHAL O. and JANN K. (1965): Bacterial lipopolysaccharides. Extraction with phenol-water and further application of the procedure. Methods Carbohydr. Chem., **5**, 83–91.
28. WOLLENWEBER H. W. and RIETSCHEL E. T. (1990): Analysis of lipopolysaccharide (lipid A) fatty acids. J. Microbiol. Methods, **11**, 195–211.

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