STUDY OF NITRIC OXIDE PRODUCTION BY MURINE PERITONEAL MACROPHAGES INDUCED BY BRUCELLA LIPOPOLYSACCHARIDE

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Abstract - Brucella is a gram negative bacteria that causes Brucellosis. Lipopolysaccharide (LPS), the pathogenic agent of Brucella, is composed of O-chain, core oligosaccharide and lipid A. In addition, the structural and biological properties of different LPS extracted from different strains are not identical.

The first defense system against LPS is nonspecific immunity that causes macrophage activation. Activated macrophages produce oxygen and nitrogen radicals that enhance the protection against intracellular pathogens.

In this experiment LPS was extracted by hot phenol-water procedure and the effect of various LPSs on nitric oxide production by peritoneal mouse macrophages was examined.

Our results demonstrated that the effect of LPS on nitric oxide production is concentration-dependent and we observed the maximum response in concentration of 10-20 microgram per milliliter. Also our results demonstrate that LPS extracted from vaccine Brucella abortus (S 19) had a higher effect on nitric oxide production than the LPS from other strains.

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Key Words: Brucella abortus, brucella melitensis, LPS, peritoneal macrophage, nitric oxide

INTRODUCTION

The members of the genus Brucella are gram-negative bacteria pathogenic for both animals and humans. Presently several species, which differ in host, surface antigens and other phenotypic and genotypic features are recognized (1,2). Two of the most common species, B. melitensis and B. abortus, bear the antigenic characteristic of smooth (S)-type- lipopolys-accharide (S-LPS) and elicit a strong immunological response in their natural hosts. The immuno-dominant molecule involved in their response is LPS (3,4). The chemical composition of Brucella LPS considerably differs from that of enterobacterial LPS. LPS generally contains O-chain, core oligo saccharide and lipid A (5). O-chain of S-LPS of Brucella is an un-branched homopolymer from about 10 to 100 units of 4-formamido-4, 6-dideoxy-D-manno pyronosyl residues, linked to a core oligosaccharide. The lipid A contains 2,3-diamino-2,3-dideoxy-D-glucose as backbone (4,6).

In contrast to many endotoxins, Brucella LPS is non-pyrogenic, does not activate complement to any significant level and is very weak mitogen for murine B cells and is a weak inducer of IFN-γ production. However, Brucella LPS stimulates the production of interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α) (5,7,8). On the other hand, LPS from gram-negative bacteria are potent activators of nitric oxide synthase (NOS) which generate nitric oxide (NO) from arginine (9,10). Also , Brucella LPS induces production of NO in rat peritoneal macrophages although such an effect is significantly lower than Eschericia coli (E. coli) LPS. These observations might explain :1. the acute outcome of Brucella infection; 2. the low frequency of septic shock in human Brucellosis; 3. the prolonged intracellular survival of Brucella in humans (11-13).

NO was identified as the effector molecule in killing a wide range of intracellular pathogens (13). Abundant evidence suggest that macrophages kill or inhibit the growth of tumor cells, bacteria, fungi, and parasites (9). While NOS gene is turned on in macrophages, these cells can eliminate a wide range of intracellular pathogens. NO production is induced by LPS and its production can be increased by IFN-γ and TNF-α but repressed by glucocorticoids, transforming growth factor -β (TGF-β), interleukin-4 (IL-4) and interleukin-10 (IL-10) (9).

In the present study, we investigate the effect of LPS extracted from four different Brucella strains on induction of NO by Balb/c mice peritoneal macrophages in order to see whether there is any biological difference between these strains.

MATERIALS AND METHODS

Bacterial strains: B. abortus biotype 1, B. abortus S19 (vaccine strain), B. melitensis biotype 3, and B. Melitensis Rev 1 (vaccine strain) were kindly provided by Dr. Zowghi from Razi Institute, Hessarak, Karaj, Iran.

Mice: 6-8 week old female Balb/c mice were purchased from Razi Institute, Hessarak, Karaj, Iran.

LPS extraction: LPS extracted by hot phenol-water procedure. Brucella crude S-LPS was obtained by methanol precipitation of the phenol phase of a water-phenol exactly as described previously (14). In order to further purify the LPS fraction, nucleic acid and protein contamination was eliminated by adding nuclease (50 μg/ml), lysozyme (25 μg/ml) and proteinase K (50 μg/ml). Subsequently the mixture was incubated for 48 h at room temperature. The purified LPS was lyophilized and kept at 4-8 °C until used (15-16).

SDS-Polyacrylamide electrophoresis (SDS-PAGE) was performed based on Lammeli method. 10 μg of each LPS subjected to the electrophoresis (17). For visualizing the results silver staining was used (17).

KDO assay: The LPSs were hydrolyzed in 0.02 N H_2SO_4 at 100°C for 20 min and subsequently treated with HIO_4 and $NaHSO_4$. The formyl pyruvic acid thus was reacted with thiobarbituric acid (TBA) to give red chromophore which has maximum absorption at 548 nm. The KDO concentration was calculated based on the standard curve (18).

Peritoneal macrophage culture: RPMI was injected to the peritoneal cavity of BALB/c mice and the peritoneal macrophages were isolated and collected after a few minutes. The cells were then cultured in complete medium consisting of RPMI, 10% heat-inactivated feotal bovine serum, 2 mM L-glutamine, 100 μg/ml streptomycin and 100 IU/ml penicilin at the density of 2 $\times 10^6$ cells/ml. 200 μL of the cell suspension was dispensed into each well of the 96 well culture plates which were then incubated at 37°C in humidified atmosphere with 5% CO_2 and 95% air. The cells were then stimulated by different doses of each extracted LPSs and incubated for 24 h. The supernatants were collected to measure No production using Griess's method (19,20).

Griess reaction: Briefly, the supernatants mixed with equal volums of 1% sulfanilamide in H_3PO_4 and 0.1% naphtyl-ethylene diamine dihydro chloride in H_2O. The reactions completed at room temperature within 5-10 min and the optical density of each reaction mixtures was read at 550 nm. NO production was measured based on the standard curve of sodium nitrate (10,21).

Statistical analysis: student t test was used to see the difference of NO production induced by different LPSs and P value 0.05 was considered significant.

RESULTS

SDS-PAGE: SDS - PAGE of all LPSs were identical as shown in Fig. 1. Two smear-like regions were observed. The first one had low mobility and consisted of complete LPS and the second region had high mobility and consisted of lipid A and the core.

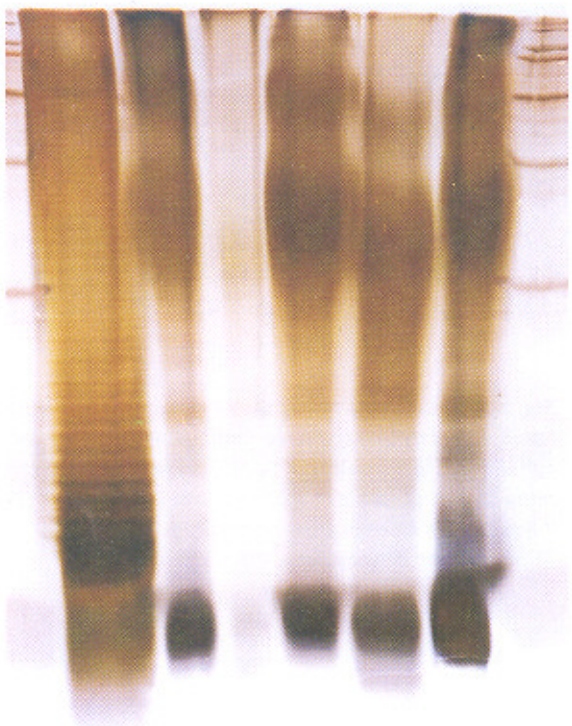

Fig. 1. SDS-PAGE pattern of LPS from different strain of Brucella One smear with high molecular weight appears in the top of the gel and one smear with low molecular weight appears in the bottom. 1 ;Salmonella LPS, 2; Rev-1 LPS, 3;BM-1 LPS, 4;S19 LPS, 5;BA-3 LPS.

KDO analysis: Under the explained experimental conditions, less than 1% by weight of all LPSs consists of KDO.

NO productions: LPS concentrations used throughout this study ranged from 5 to 30 μg/ml. As shown in figure 2, LPSs induced NO production in dose-dependent manner and the optimum production of NO were seen by 10 μg/ml of each LPSs. The highest production of NO was seen by 20 μg/ml of BA-3 and S-19. Production of No was reduced at 30 μg/ml of each LPSs. LPS extracted from S19 was

shown to be more potent than LPS extracted from B. abortus (biotype 3) Fig. 3. In addition, LPS extracted from Rev 1 was potent inducer of NO compared to LPS extracted from B. melitensis (biotype 1), (Fig. 4). However the potency of LPS extracted from S19 was significantly ($p<0.05$) higher than Rev 1 (Figures. 3,4)

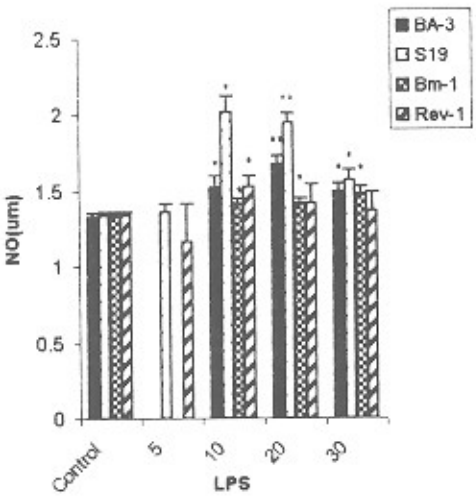

Fig. 2. NO production by peritoneal macrophages of Balb/c mice induced by LPS extracted from four Brucella strains. 2 10^6 cells/ml were cultured in the presence of different concentration of each LPS and incubated for 24h.
* Significantly different from control $P < 0.001$
** Significantly different from control $P < 0.0001$

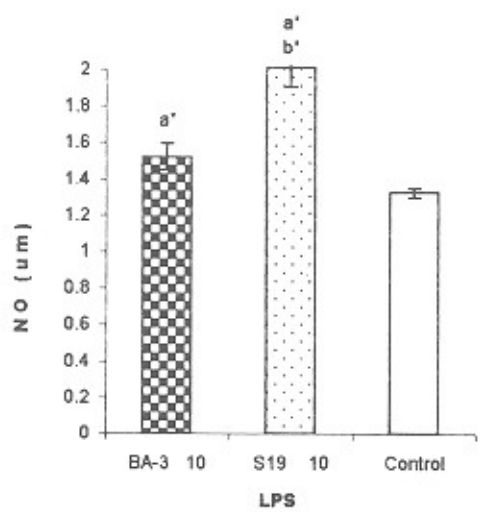

Fig. 3. Comparative demonstration of NO production at the optimum dose of LPS extracted from two strains of B.abortus, the vaccine strain (S 19) and a pathogenic, field isolate (BA-3).
a* Significantly different from control ($P < 0.001$).
b* Significantly different S19 from BA-3 ($P < 0.0001$).

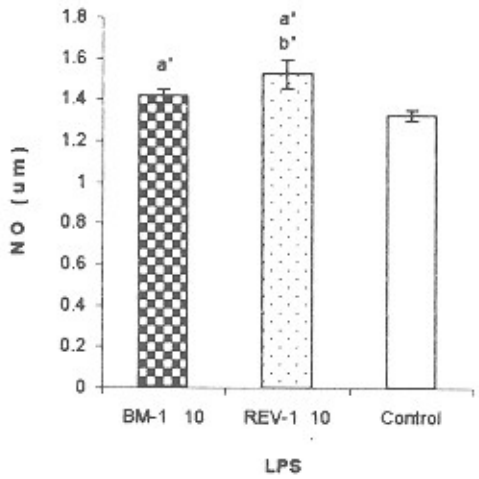

Fig. 4. Comparative demonstration of NO production at the optimum dose of LPS extracted from two strains of B melitensis. , the vaccine strain (Rev-1) and a pathogenic, field isolate (Bm-l).
a* Significantly different from control ($p<0.001$).
b* Significantly different BM-l from Rev-l ($p<0.01$).

DISCUSSION

SDS-PAGE has been used for characterization of LPS (17). The high molecular weight aggregates of LPS dissociate into monomeres complexed with SDS due to hydrophobic binding of SDS to the lipid A. The slow migrating component which appeared as a smear is caused by the presence of the O-chain with variable number of single repeating units (22,6). It has been shown that SDS-PAGE is not able to show the heterogeniticity between different LPSs to Brucella strains (6,22,23). In the present study, LPSs extracted from different strains showed similar SDS-PAGE pattern which is in consonance with previous studies (6,17,22). KDO component of the extracted LPSs was less than 1% which is similar to the previous reports (18). It has been demonstrated that most of the biological activities induced by Brucella are quantitatively and qualitatively dependent on its LPS which are related to the unique structure of this molecule (23,25-32). However, there are very limited studies pertaining the effect of Brucella LPS on NO production relating to the state of the infection (13). We showed that the effect of LPS on NO production by murine peritoneal macrophages is dose and strain dependent. The peak production of NO has gained in 10 μg/ml for all LPSs. Dr. Lopez-Urrutia , L., (11) also found that the same concentration of LPS 10 μg/ml induced optimum level of NO production on peritoneal macrophages of rat. We can show that S19 was the

most potent inducer of NO in murine peritoneal macrophages. This is the first demonstration of NO production by S19 LPS. Since NO is involved in the clearance of the infection (13), LPS would play an important role in induction of NO protection against the disease. Brucella LPS has been introduced as candidate vaccine and its effect on IL-12 production has been documented (24,22). In conclusion, we demonstrated that the potency of various Brucella LPS is different and S19 is the potent inducer of NO and might be the best candidate vaccine.

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