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Immuno-pathophysiological responses of mouse model to experimental infection with *Brucella melitensis* and its lipopolysaccharides via intraperitoneal route

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ABSTRACT

Brucella melitensis is one of the major zoonotic pathogens with significant economic implications worldwide. The pathogenicity is complex and not always well understood. Lipopolysaccharide (LPS) remains the major virulent factor of *B. melitensis* and responsible for the mechanism by which the pathogen causes its deleterious effects. In this study, 84 mice of 6–8 weeks old of both sexes were divided equally into 3 groups; namely *Brucella melitensis* infected group, lipopolysaccharide (LPS) infected group and control group. The former two groups contained 36 mice each with equal gender distribution. The control group consisted of 12 mice only. Animals in *B. melitensis* infected group, a single inoculum of 0.4 ml containing 10^9 of *B. melitensis* were intraperitoneally challenged while animals in LPS group, a single dose of 0.4 ml containing LPS extracted from the *B. melitensis* were intraperitoneally inoculated. Animals in control group received intraperitoneally, a single dose of 0.4 ml phosphate buffered saline (PBS) of pH7. Animals that were infected intraperitoneally with *B. melitensis* demonstrated significant clinical presentation; gross and histo-pathological evidence than LPS infected group. However, both infected groups showed elevated levels of interleukins (IL-1β and IL6), antibody levels (IgM an IgG) as early as 3 days post-infection with predominance in LPS infected group. In contrast, low levels of sex related hormonal changes in which LPS infected group showed the least concentration were also detected throughout the experimental period. In conclusion, *B. melitensis* can be transmitted via gastrointestinal, respiratory and reproductive tract. Moreover, LPS stimulated significantly the innate and acquired immune system without significant systemic dysfunction, suggesting potentiality of the protective properties of this component as alternative vaccine for brucellosis infection.

1. Introduction

Brucella melitensis is the major zoonotic pathogen with significant economic implications in animal industry worldwide [34]. The pathogen is able to infect a large diversity of mammalian hosts with special predominance in small ruminants and cattle causing abortion and reduced fertility [12,18,27]. *B. melitensis* has long been believed to be the most virulent pathogen among the zoonotic species of the genus *Brucella* causing devastating epidemics with high morbidity and mortality in Mediterranean Basin, Middle East, Africa, Latin America and central Asia [6,37]. Infections are believed to occur by ingestion, inhalation or direct contact of the organism with broken skin or mucous membranes, venereal transmission and latent infection of neonates [8]. Higher incidence of *Brucella melitensis* is associated with environmental and management factors which include moist, humid conditions, high animal population density, extensive free grazing system and poor husbandry practice [9].

The disease is characterized as fertility related issue with clinical signs such as abortions during the trimester, retained placenta, metritis, placentitis, orchitis, epididymitis in which the lipopolysaccharide is a major part of the disease pathogenesis [8,23].

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Infection is generally believed to establish through different routes which include intraperitoneal, oral, respiratory, subcutaneous and conjunctiva resulting in septicemia with isolation of the organism from several organs [15]. However, circumstantial evidence suggests the involvement of multisystem in the pathogenesis of *Brucella melitensis* in animal models [8,15]. Intraperitoneal infections in laboratory animals have long been described to be a suitable route of infection in terms of producing maximum levels of infection compared to other routes [15]. Substantial evidence have also indicated that this route is preferable because of its simplicity, reliability and reproducibly as it admits a larger volumes and, therefore, less prone to inoculation errors [19]; Nevertheless, the pathogenesis and the immunopathophysiology of the *B. melitensis* and its lipopolysaccharide following intraperitoneal infection have not been well documented since previous reports were limited to incidental observations [15,21].

2. Materials and methods

2.1. Ethics statement

All experiments were conducted according to protocols reviewed and approved by the Institutional Animal Care and Use Committee (IACUC), Faculty of Veterinary Medicine, Universiti Putra Malaysia (UPM) with reference no. UPM/IACUC/AUP-R049/2015.

2.2. Animals

A total of 84 mice, both sexes, six-to-eight weeks-old BALB/c strain were used in this study. The mice were purchased from the Laboratory Animal Services Centre, Malaysia, bred in individually ventilated cage rack systems, and subsequently transferred to the facilities of Universiti Putra Malaysia at the beginning of the experiments. Mice were acclimatized for a period of two weeks and provided *ad libitum* access to water and pellets throughout the experimental period.

2.3. Experimental design

All the mice were randomly assigned into three major groups; namely *B. melitensis* treated (n = 36), LPS treated (n = 36) and control group (n = 12). The former two groups consist of 36 mice with equal gender distribution each (18 female + 18 male). However, the control group consisted of 12 mice. Briefly, animals in *B. melitensis* group, single inoculum of 0.4 ml containing 10^9 of *B. melitensis* was intraperitoneally challenged while animals in LPS group, a single dose of 0.4 ml containing 10^9 lipopolysaccharide extracted from *B. melitensis* was intraperitoneally inoculated. Animals in control group, however, a single dose of 0.4 ml phosphate buffered saline (PBS) of pH7 was used. All mice were observed at 12 h, 48 h, 72 h and then at 7 days interval for clinical signs, necropsy and histopatholgic evaluation as well as blood analysis for antibody, cytokine and steroid hormone levels for up to 24 days post-infection.

2.4. Synchronization

Induction and synchronization of oestrus and ovulation in mice were performed with slight modification as previously described [31]. A total of 42 BALB/c females, divided into three groups were considered. These female mice were treated with two intraperitoneal doses of 0.5 μg of cloprostenol on Days −3 and 0 day.

2.5. Bacterial strain and media

A stock culture of *B. melitensis*, an epidemic strain that previously isolated from an outbreak in Malaysia was used to prepare the inoculums. All bacteria were routinely grown on trypticase soy broth (TSB) or *Brucella* agar which contains growth supplements; namely biotin, thiamin, and nicotinamide. The optimum growth temperature is 36 °C–38 °C whereby the pure colonies of *B. melitensis* can be visible after 4 days of incubation period. Thereafter, the bacteria were suspended and diluted in sterile phosphate buffered saline (pH 7) containing 0.5% Tween 80. All work with the virulent *Brucella* strain was performed in biosafety level 3 facilities, Universiti Putra Malaysia (UPM).

2.6. Inoculum preparation of Brucella melitensis

Throughout the experiment, two types of the *inocula* were used; the whole cell of *B. melitensis* and its lipopolysaccharide (LPS). The organisms were cultivated during 24 h on the surface of *Brucella* agar medium. The culture was harvested in diluent; suspensions of bacteria were standardized turbidimetrically and adjusted to the required concentration of 10^9 of *B. melitensis* by appropriate dilutions using McFarland Nephelometer Barium Sulfate Standards.

2.7. LPS extraction from B. melitensis

The lipopolysaccharide (LPS) of *B. melitensis* was extracted using Intron Biotechnology® LPS Extraction Kit. In this study, 10^9 CFU of *B. melitensis* was used for LPS extraction. In this, the bacteria were harvested by centrifugation in room temperature at 13,000 rpm for 30 s. Then, one ml of lysis buffer was added and the mixture was vortexed vigorously. Of 200 μl chloroform was later added and again it was vortexed for 10–20 s before it was incubated for 5 min at room temperature. Following that, it was centrifuged at 13,000 rpm for 10 min at 4 °C and the supernatant was transferred into a new 1.5 ml appendorf tube. Thereafter, 800 μl of purification buffer was added to the supernatant and mixed well before it was incubated at −20 °C for 10 min. Following this, it was centrifuged at 13,000 rpm for 15 min at 4 °C. The LPS pallet was obtained after the excess supernatant was discarded before the LPS pallet was washed with 1 ml of 70% ethanol, which was then dried completely. Finally, 70 μl of 10 mM Tris-HCl buffer of pH 8.0 was added to the LPS pallet and allowed to dissolve by boiling for 2 min.

2.8. Route of exposure

2.8.1. Intraperitoneal inoculation

Animals in *B. melitensis*, a single inoculum containing 0.4 ml of 10^9 CFU of *B. melitensis* was administered intraperitoneally using a 30-gauge needle while animals in LPS group received a single inoculum containing 0.4 ml of 10^9 CFU of LPS extracted from *B. melitensis.* Similarly, animals in control group, challenges were intraperitoneally administrated using a single inoculum containing 0.4 ml of phosphate buffered saline (PBS).

2.9. Clinical observation

Clinical observations were recorded twice daily for all animals throughout the experimental period. All tested parameters were documented according to the protocols described by Xavier et al. [39].

2.10. Gross pathology

Necropsy was conducted immediately after euthanasia. The