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PLGA (85:15) nanoparticle based delivery of rL7/L12 ribosomal protein in mice protects against *Brucella abortus* 544 infection: A promising alternate to traditional adjuvants

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ABSTRACT

There is a compelling need for the development of suitable adjuvants for human use to enhance the efficacy of the upcoming vaccines for the prevention of life threatening infections. In the current study, we have tried to explore the immunogenic potential of nanoparticles (NPs) made of PLGA (poly lactic-co-glycolic acid), a biodegradable and biocompatible polymer approved by FDA for human use after entrapping rL7/L12 protein, an immunodominant antigen of *Brucella*. Adjuvant properties were exhibited by the formulation as it elicited high IgG antibody titers just after first immunization which increased significantly after the booster administration. A good elicitation of the Th1 cytokines especially IFN-γ was recorded. Amongst the IgG antibody subclasses, IgG1 remained the predominant subclass to be elicited in mice serum after immunization; however IgG1/2a ratio showed a mixed profile of Th1/Th2 response. Lymphocyte proliferation assay as a marker of amplification in cellular immunity demonstrated that the splenocytes of the immunized mice had a high proliferation index with reference to the control, revealing that L7/L12 entrapping PLGA nanoparticles are potent inducer of inflammatory cell response indispensable to combat *Brucella* infection. Enumeration of splenic CFU after 14 days of infection with *Brucella abortus* 544 showed a significant reduction in log CFU of splenic bacteria in the vaccinated mice as compared to the control group. Therefore it is evident that PLGA nano formulations delivering the entrapped vaccine candidate in mice elicit specific humoral as well as cellular responses specific to the entrapped *Brucella* antigen. So there is much promise in this approach and this work by highlighting the adjuvant properties of the PLGA nanospheres will accelerate the development of improved vaccines safe for human as well as veterinary use.

1. Introduction

Brucellosis is the most common bacterial zoonosis in the world causing over 500,000 new human cases annually (Pappas et al., 2006). *Brucella*, the etiologic agent of Brucellosis belongs to the family Brucellaceae under order Rhizobiales of Alpha proteobacteria (Moreno et al., 1990). These are gram negative, non motile and non spore forming coccobacilli. The zoonosis affects a wide variety of hosts including humans. Till date several species of *Brucella* have been isolated from a range of host species including goats, sheep, cattle, swine, dogs, dolphin, porpoise, whale, seal, desert wood rat, common vole as well as human patients (Gomez et al., 2013). In animals, *e.g.*, cow, buffalo, sheep, goat, swine etc. it causes abortions and infertility (Godfroid et al., 2005; Renukaradhya et al., 2002) while in humans, it evokes undulant fever, endocarditis, osteomyelitis and arthritis which may get extremely severe upon prolonged infection finally culminating in conditions worse to death (Pappas et al., 2003).

Brucella, being facultative intracellular pathogen, perpetuates inside the monocytes, macrophages and dendritic cells of the host by avoiding phagosome–lysososome fusion (Baldwin and Goenka, 2006). In contrast to other pathogenic bacteria it does not have any classical virulence factors, such as exotoxins, endotoxic lipopolysaccharide, cytolysins, capsules, etc. therefore its infection remains stealthy, not marked by a provoked inflammatory immune responses or cytokines (Wernery, 2014; Seleem et al.,

Abbreviations: PLGA, poly lactic-co-glycolic acid; FDA, food and drug administration; APCs, antigen presenting cells; CMI, cell mediated immunity; CFU, colony forming units; TSA, tryptic soya agar; NPs, nanoparticles; DLS, differential light spectroscopy.

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2010). Therefore the infection slowly progresses to chronic stages without enough host immune resistance.

Considering the re-emergence of the disease throughout the world and its increasing spread, it becomes imperative to discover new therapeutic as well as prophylactic tools to counter the disease occurrence. Prevention for sure remains the preferred and safe option. In this direction, there are a lot of live attenuated vaccines, *e.g.*, *B. abortus* S19, *Brucella melitensis* Rev1, *B. abortus* RB51, *B. melitensis* M5 that have been discovered and extensively used (Saez et al., 2014) but all are applicable to animals and none for human use apart from being associated with a certain level of risk upon administration (Moriyon et al., 2004). All the live vaccines suffer from the danger of reversion to full virulence and pathogenicity; can cause abortions if administered to pregnant cattle and all of them are pathogenic to humans so they are a health risk to the administering personnel as well. Furthermore, most if not all these vaccines, create a diagnostic confusion because of eliciting sera positivity against the *Brucella* LPS, which is the main component of the most common popular lateral flow/ELISA based diagnostic kits. Therefore, development of a safe subunit vaccine to prevent Brucellosis is gaining impetus; introducing the use of several potent antigens and their combination as well as different types of adjuvants and delivery vehicles *e.g.*, micro or nanoparticles to the field of *Brucella* vaccine development.

PLGA (poly lactide-*co*-glycolide) in itself is a co-polymer of lactic and glycolic acid (biodegradable), which can be synthesized in different sizes based on the process used. PLGA has been shown to possess adjuvant properties as a polymer but as particulate nanospheres it has been found to be taken up by the antigen presenting cells of the host and owing to this property it is used to target the immunogenic cargo into the macrophages and dendritic cells of the host causing huge antigen presentation facilitated by undiluted or high content of antigen internalization that occurs indeed (Greenland and Letvin, 2007; Soppimath et al., 2001)

In the current study, we have entrapped only rL7/L12 protein, a ribosomal protein of *B. abortus* and an immunodominant antigen into PLGA nanoparticles not using any other available adjuvants to check the immunogenicity conferred in Swiss albino mice after administration in a prime-boost regimen. The sera titration results show good adjuvanticity of PLGA with the protein by giving high IgG titer profile after single immunization. *In vivo* protective efficacy was assessed by estimating bacterial CFU in the spleens of mice immunized with nanovaccine formulation and a significant reduction in the bacterial burden was observed. The overall immune response ensued after vaccine administration, humoral *vs.* cellular was assessed to evaluate the corresponding reasons for clearance of the pathogen.

2. Materials and methods

2.1. Materials

PLGA polymer (poly lactide-*co*-glycolide; 85:15), dicloromethane (DCM), poly vinyl alcohol (PVA) were procured from Sigma. Anti mouse antibodies and HRP-linked secondary antibodies were procured from Santacruz.

2.2. Animals, bacterial strains and plasmids

B. abortus S19 and *B. abortus* 544 have been obtained from IVRI Izzatnagar. Female Swiss albino mice weighing 28.2 ± 0.85 g (6–8 weeks old) were used for all immunization studies. *Escherichia coli* BL-21 λ DE3 has been used along with DH5 alpha for propagating the plasmids.

2.3. l7/l12 *gene cloning*

Coding sequence of *l7/l12* gene (396 bp) of *B. abortus* was cloned in pET28a vector at *Nco*I/ *Xho*I restriction sites for transformation in DH5α strain of *E. coli* (Supplementary Fig. 1).

2.4. *Protein expression and purification*

Protein purification was performed by Ni^{+2}-NTA affinity column using cytosolic fraction of the BL-21λ DE3 expression cells. Dialysis was done against PBS (pH 7.4) and protein was quantified and concentrated for entrapment and further immunization.

2.5. *Formulation of potential vaccine in chemically stable PLGA NPs*

PLGA (lactide:glycolide = 85:15) (Sigma–Aldrich, U.S.A.) nanoparticles were prepared using double emulsion (W1/O/W2) solvent evaporation technique according to the protocol in the mentioned reference (Manish et al., 2013). Briefly, a w/o emulsion was first prepared by mixing the PLGA solution (organic phase) and the protein (water phase) employing probe sonication (2 mm stepped microtip) at 35% amplitude and 60 s (750 W Sonic Vibra cell sonicator). Subsequently, the mix was then poured in 1% Poly vinyl alcohol solution (outer aqueous phase) along with probe sonication using 6 mm stepped tip at 30% amplitude for 110 s to get the final water/oil/water emulsion. To get rid of the DCM (dichloromethane) present in the organic phase the solution was stirred at room temperature for 6–8 h under sterile conditions. After that the solution was centrifuged at 15,000 g for 15 min and washed twice with the deionized water. The final pellet was suspended in double autoclaved water and lyophilized for 16–18 h at 0.003 mBar pressure and −43 °C temperature. No cryoprotectants were used and the fine white powder obtained was stored in −20 °C. Lyophilized powder (5 mg) of nanoparticles was taken; incubated in 1 ml of acetonitrile for 30 min, centrifuged at 16,000 RCF for 15 min, the supernatant obtained thereof was discarded and the pellet was dissolved in 40 μl of 1% SDS and analysed by SDS-PAGE to confirm the entrapment visually.

2.6. *Physical characterization of NPs and their* in vitro *release study*

The size of the nanoparticles was determined by DLS (differential light spectroscopy) while its shape and surface morphology was analysed by Zeiss EVO40 Scanning electron microscope (Carl Zeiss, Thronwood, NY); surface charge was measured by Zeecom zeta potential analyzer. The particles (dissolved in PBS) were kept at 37 °C for different time periods varying from 1 day till 28 day after which the sample was centrifuged at 10,000 rpm and the micro-BCA was performed using 150 μl of supernatants in triplicates and the absorbance was recorded at 562 nm to assess the protein released during that period and mean with SD was calculated. Further the total yield of the PLGA Nps was calculated by using the formula {(the total weight of the dried PLGA Nps formed/total weight of the PLGA polymer (85:15, Mw 50,000–75,000) employed for making the Nps) × 100%}. The antigen load present in 1 mg of the Nps was estimated by the micro-bichinchoninic acid method.

2.7. *Immunization experiments*

6–8 weeks old swiss albino mice (female; 28.2 ± 0.85 g), were used. In the experimental group, each mice was administered with the required amount (31.2 mg) of nanoparticles ensuring that every mice received the same dose (40 μg) of rL7/L12, i.p. (intraperitoneal). Blood samples were taken at days 0, 14, 28, 35 and 42 and