Vaccine 36 (2018) 1837–1845

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Safety implication of *Salmonella* based *Brucella* vaccine candidate in mice and in vitro human cell culture

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ARTICLE INFO

Article history:
Received 24 October 2017
Received in revised form 15 February 2018
Accepted 16 February 2018
Available online 24 February 2018

Keywords:
Live bacterial vaccine vector
Salmonella Typhimurium delivery
Brucella
Vaccine
Human
Mice model

ABSTRACT

An anti-*Brucella* vaccine candidate comprising rough *Salmonella* vector delivering *Brucella* antigens was developed. This system provides a platform for live *Brucella*-free vaccine development as it can mimic active-intracellular infection of *Brucella* organism. Exploiting this phenomenon thus provides significant protection at a single dose and also re-assured the safety. To date, no human anti-*Brucella* vaccines are available, owing to the lack of safe and effective formulation. This study investigated the safety of the vaccine formulation in mice model and in vitro human cell cultures. The experiment was designed to determine the LD50 of the vaccine formulation. The vaccine formulation did not induce any mortality even when mice were administered at 8×10^9 CFU per oral or per subcutaneous (SC), which was 100-times more than the actual vaccine dose intended for mice model. In contrast, wild-type (WT) *Salmonella* positive control strain induced 100% mortality at 8×10^7 CFU per mice via oral or SC routes. Interaction of the vaccine with phagocytic (THP-1 derived macrophage) and non-phagocytic (Caco-2) human cell lines as well as human PBMC was investigated. In in vitro experiments, inflammatory and pyretic cytokines TNF-α, and IL-1β inductions were significantly lower in vaccine group as compared to WT group. Further, apoptosis, nitric oxide synthase and cytotoxicity inductions were comparable and not exacerbated, given that the strain is based on a rough bacterial vector that may have endotoxic lipid-A more readily exposed. These findings corroborated that the vaccine formulation is highly safe in mice model and is relatively mild in the induction of inflammatory cytokines and cellular changes in human cell lines.

1. Introduction

Attenuated *Salmonella* delivering *Brucella* antigens provided a platform for the development of safer-effective vaccine candidate. In the absence of a suitable human anti-*Brucella* vaccine, research efforts are directed towards safer inactive, subunit vaccines, or vaccine vectors that avoid application of *Brucella* organism. In our previous study, we have reported the development of anti-*Brucella* vaccine candidate which was based on a *Salmonella* Typhimurium vector delivering immunogenic *Brucella* proteins [1]. The *Salmonella* delivery vector JOL1800 is a lipopolysaccharide (LPS) biosynthesis gene "rfaL" (O-antigen ligase) knocked out mutant (ΔrfaL) with rough phenotype derived from the original JOL912 strain via lambda red recombination [1]. The strain JOL912 is a triple mutant (Δ*lon*Δ*cpxR*Δ*asd*), with smooth phenotype derived from a wild-type *Salmonella* strain (JOL401). The vaccine formulation designated as RSrBL (rough *Salmonella* delivering recombinant *Brucella* antigens) comprises of a cocktail of four *Salmonella* strains, each delivering *Brucella* antigens namely, superoxide dismutase (SOD), *Brucella* lumazine synthase (BLS), outer membrane protein-19 (OMP19) and proline racemase protein A (prpA). The primary objective of the study is to investigate the safety implications of the vaccine candidate in the in vivo system, by determining the lethal dose 50 (LD50) of the vaccine strains in mice model and investigate the interaction with the cultured human cells.

Several authors have reported that LPS of Gram-negative bacteria plays a critical role in initiating endotoxic shock and sepsis [2,3]. However, our understanding pertaining to the lack of O-antigen in a delivery vector and yet the induction of inflammatory response is incomplete. *Salmonella* vector with truncated LPS may be more prolific in the induction of sepsis, as it can be presumed that the deficiency of O-antigen side chain may result in increased exposure of the lipid A component of the LPS to the external surface and to immune cells. In this study, we have selected mediators that are biomarkers of sepsis such as tumor necrotic factor-α (TNF-α), interleukin-1 (IL-1) and nitric oxide (NO) radical productions [4]. Macrophages play a major role in generating the response

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https://doi.org/10.1016/j.vaccine.2018.02.069
0264-410X/

against endotoxins. Nitric oxide production and TNF-α secretion by these cells have been proposed as the primary mediators of this event. The adverse effects of LPS in an event of endotoxic shock are associated with oversecretion of TNF-α and NO both predominantly released by tissue macrophages [5]. With this background information, THP-1 derived macrophages were used for the in vitro studies. THP-1 is an immortalized monocyte-like cell, derived from the peripheral blood of a boy with acute monocytic leukemia [6]. THP-1 is commonly differentiated to a macrophage via treatment with phorbol 12-myristate 13-acetate (PMA). A non-phagocytic human Caco-2 cell line was also selected for the safety-related study. The Caco-2 cell line is a continuous cell of heterogeneous human epithelial colorectal adenocarcinoma cells [7]. Further, human primary monocyte culture-peripheral blood mononuclear cells (PBMC) were also included in the cytokine induction study. In summary, we have investigated the effects of inoculation with regard to host safety issues in in vivo mice model. We also investigated the induction of apoptosis, intracellular nitric oxide synthase (iNOS), cytotoxicity, and induction of inflammatory cytokines in human cell lines.

2. Materials and methods

2.1. Ethics and biosafety statement

All animal experimental procedures were approved (CBNU2015-00085) by the Chonbuk National University Animal Ethics Committee in accordance with the guidelines of the Korean Council on Animal Care and Korean Animal Protection Law, 2007; Article 13 (Experiments with animals). All mice used in the study were housed and maintained humanely and were provided water and antibiotic-free food *ad libitum*. The human PBMC was isolated from blood samples (O+ and B+ types) donated by Red Cross Korea (Reference No: 10-17-029914 and 10-17-029642, respectively).

2.2. Bacterial strains, plasmids, and primers

The bacterial strains, plasmids, and primers used are listed in Table 1. All *Salmonella* strains and *E. coli* strains were grown in Luria-Bertani (LB) medium. For the expression of *Brucella* antigen in *Salmonella* strain, open reading frame (ORF) of the candidate gene was cloned into pJHL65 plasmid using *EcoRI* and *HindIII* restriction sites [1]. Vaccine cocktail was prepared in phosphate buffered saline (PBS) constituting an equal number of colony forming units (CFU) to meet the required CFU dosage.

2.3. Lethal dose 50 determination of the vaccine candidate and histopathological analysis

A total of 48 4-week-old specific pathogen free (SPF) female BALB/c mice were divided equally into 8 groups (n = 6/group), fasted for 20 h, and were inoculated via oral or subcutaneous (SC) routes with a ten-fold serial dilutions (8×10^7, 8×10^8 and 8×10^9 CFU's) of an inoculum comprising equal CFU of the four vaccine strains listed on Table 1 (JOL1878, JOL1879, JOL1880 and JOL1881). For reference and lethality control, two mice groups were also inoculated with the virulent wild-type strain (JOL990) at a dose of 8×10^7 CFU via oral and SC routes. The mice were then observed twice daily for vaccine-related safety assessment (i.e., observation for any morbidity and mortality; mice body weight determination). Organs samples for tissue sections were harvested promptly in buffered 10% formalin upon mortality of the inoculated mice or at the end point of the experiment. The experiment was conducted for 20 days postinfection and the remaining surviving mice were humanely euthanized at the completion of the experiment.

Histopathological assessment was performed as per the procedure described earlier with modification suitable for mice [8]. Tissues sample sections were derived from organs of the mice following inoculation. Lungs, liver, and spleen were processed for tissue sectioning and subjected to hematoxylin-eosin (HE) staining. Additionally, organs of three naïve mice were also processed and used as a reference normal control. The histopathological slides were examined using stereo-microscope (Leica Microsystems, Germany) in order to determine any alterations induced due to the experimental infections.

2.4. In vitro human cell cytotoxicity assay

The in vitro cytotoxic effect of the bacterial strains on THP-1 derived macrophages and Caco-2 human cells were investigated. The population of dead cells was quantified using Incucyte™ Cytotox Green reagent and counting was carried out using Incucyte™ ZOOM (Essen BioScience, US). The experiment was performed according to the manufacturer's recommendation. Briefly, 0.5×10^6 THP-1 or 4×10^5 Caco-2 cells were seeded in 96 well plates in replicates. For THP-1 cells, differentiation from monocytes to macrophage was achieved via treatment with 10 nM/ml of phorbol 12-myristate 13-acetate (PMA). The cultures were twice treated with PMA and incubated for 5 days. Upon observation of morphological changes, the macrophages were treated with 10 multiplicity of infection (MOI) of WT JOL990 or vaccine cocktail. Caco-2 cells

Table 1
Bacterial strains, plasmids, and primers used in this study.

Strain/plasmid/primer	Description	Reference
X232	*E .coli* Δ*asd* strain, used for cloning of genes into *asd*$^+$ plasmid	Lab stock
JOL990	Smooth *Salmonella* Typhimurium wild type; SPI-1 *invAE*$^+$ *hilA*$^+$ *avr*$^+$; SPI-2, amino acid permease; SPI-3, *mgtC*$^+$; SPI4, ABC transporter; SPI5, *pipB*$^+$; reference strain	Lab stock
JOL1800	Rough *Salmonella* Typhimurium Δ*lon*, Δ*cpxR*, Δ*asd*, Δ*rfaL;* bacterial delivery vector strain	Lab stock
JOL1878	JOL1800 delivering heterologous *Brucella abortus* antigen, SOD	[1]
JOL1879	JOL1800 delivering heterologous *Brucella abortus* antigen, Omp19	[1]
JOL1880	JOL1800 delivering heterologous *Brucella abortus* antigen, BLS	[1]
JOL1881	JOL1800 delivering heterologous *Brucella abortus* antigen, PrpA	[1]
Plasmids		
pJHL65	*asd* $^+$ plasmid, pBR ori, β-lactamase signal sequence-based periplasmic secretion plasmid, 6xHis, high copy number plasmid, *Brucella* antigen cloning site	[14]