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Research paper

Inhibition of *Salmonella*-induced apoptosis as a marker of the protective efficacy of virulence gene-deleted live attenuated vaccine

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ABSTRACT

Vaccination is one of the best protection strategies against *Salmonella* infection in humans and chickens. *Salmonella* bacteria must induce apoptosis prior to initiating infection, pathogenesis and evasion of host immune responses. In this study, we evaluated the efficacy of vaccinating chickens against *Salmonella* Enteritidis (SE) using a vaccine candidate strain (JOL919), constructed by deleting the *lon* and *cpxR* genes from a wild-type SE using an allelic exchange method. In present study day old chickens were inoculated with 1×10^7 cfu (colony forming unit) of JOL919 per os. We measured cell-mediated immunity, protective efficacy and extent of apoptosis induction in splenocytes. Seven days post-immunization, the number of CD3+CD4+ and CD3+ CD8+ T cells was significantly higher in the immunized group compared to the control group, indicating a significant augmentation of systemic immune response. The internal organs of chickens immunized with JOL919 had a significantly lower challenge-strain recovery, indicating effective protection and clearance of the challenge strain. Post-challenge, the number of apoptotic cells in the immunized group was significantly lower than in the control group. Additionally, AV/PI (Annexin V/propidium iodide) staining was performed to differentiate between apoptotic cells and necrotic cells, which corroborated TUNEL-assay (terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling) results. The proportions of AV+/PI− and AV+/PI+ cells, which represent the proportions of early apoptotic and late apoptotic/early necrotic cells present, respectively, were significantly lower in the immunized group. Our findings suggest that the apoptotic splenocytes in immunized chickens significantly decreased in number, which occurred concomitantly with a significant rise in systemic immune response and bacterial clearance. This suggests that inhibition of apoptosis may be a marker of protection efficacy in immunized chickens.

1. Introduction

Salmonella Enteritidis (SE) is one of the most common *Salmonella* serotypes that cause human salmonellosis. SE is a zoonotic pathogen that commonly spreads through contaminated food, raw eggs or other poultry products (WHO databank, 2009; Matheson et al., 2010; Bäumler et al., 2000; Rodrigue et al., 1990). *Salmonella* species are facultative intracellular pathogens (Jantsch et al., 2011) that can induce macrophage apoptosis to counteract host immune responses and allow for intracellular replication (Boise and Lawrence, 2001). Inducing apoptosis allows the SE organisms to initiate infection and pathogenesis, to evade host immune responses, and to stimulate virulence through extracellular invasion (Monack et al., 1996). *Salmonella* species induce apoptosis through two major pathways: one is activated by *Salmonella*-secreted SipB protein in macrophages by way of a caspase-1-dependent mechanism (Galan, 1999; Hersh et al., 1999; Brennan and Cookson, 2000) and the other is activated by pathogen-associated molecular patterns (PAMPS) that causes macrophage cell death (Hsu et al., 2004). After ingestion of contaminated food, *Salmonella* enters the lower gastrointestinal tract and invades lymphoid follicles and the lamina propria through M cells that overlay the Peyer's patches. Once inside these lymphoid follicles, *Salmonella* activates caspase-1 via a type III secretion system inside the cytoplasm, resulting in secretion of IL-1 and macrophage cell death (Hersh et al., 1999). Macrophage cytotoxicity and killing are essential virulence mechanisms associated with invasive *Salmonella* Typhimurium (ST). These mechanisms are mediated by apoptosis and are evidenced by the nuclear morphology of damaged cells, cytoplasmic vacuolization, and host-cell DNA fragmentation (Monack et al., 1996). The importance of apoptotic mechanisms was experimentally demonstrated in mice infected with nonopsonized

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Salmonella Typhimurium, wherein bacterial loads quickly multiplied and significant macrophage death occurred; this resulted in a highly virulent infection that killed the mice within 14 days (Schwan et al., 2000).

Poultry chickens are a reservoir for SE; therefore, control of SE infection within poultry breeding and farm rearing is an important concern. Vaccinating chickens is the most widely employed strategy to control SE infection (Mastroeni et al., 2001). Routine vaccination programs have included both live-attenuated and killed vaccines, and each met with varying degrees of success. Because *Salmonella* can survive and replicate within macrophages, cell-mediated immunity is presumably required for chickens to recover from an infection (Lindgren et al., 1996; Beal et al., 2006a, 2006b; Mastroeni et al., 1993). An SE vaccine candidate strain, JOL919, was constructed by deleting the *lon* and *cpxR* genes from a wild-type SE using an allelic exchange method. The *lon* gene product has been characterized as a powerful negative regulator of the expression of invasion genes encoded on Salmonella pathogenicity island 1 (SPI-1) (Takaya et al., 2003), whereas activated *CpxR* gene regulates part of the envelope stress response system, pilus assembly, type III secretion, motility and chemotaxis, adherence, and biofilm development (Wolfe et al., 2008). JOL919 was previously evaluated for safety, generation of immunogenicity and protection efficiency against a wild-type virulent SE (Nandre et al., 2011). In previous studies, the lymphocyte proliferation response and CD45+CD3+ T cells, which are associated with activation of T helper and cytotoxic cells, were significantly augmented in the immunized group, indicating upregulation of the systemic immune responses that are required for macrophages to clear *Salmonella* (Nandre et al., 2012).

An effective vaccine candidate must induce a robust immune response that is strong enough to allow pathogen clearance. To date, there are no conclusive studies that evaluate how immunizing with live *Salmonella* vaccine affects apoptosis in splenocytes, nor co-relating it with protection. In this study, we measured how a live SE vaccine candidate, JOL919, affected the generation of cell-mediated immunity and apoptosis induction in splenocytes, as well as estimating its protection coverage.

2. Materials and methods

2.1. Selection and preparation of bacterial strains

Salmonella Enteritidis (SE) vaccine candidate, JOL919 strain, was previously constructed for immunization by Nandre et al. (2012) by deleting the *lon* and *cpxR* genes from a wild-type SE strain using an allelic exchange method. An SE virulent strain, JOL1182, was used as a challenge on four-week-old mice (Nandre et al., 2011). JOL919 and JOL1182 were individually grown overnight in Luria-Bertani (LB) broth (Becton, Dickinson and Company, Sparks, MD, USA) at 37 °C. The cultures were diluted to 1:20 in fresh LB broth and held at 37 °C to an optical density (OD) of 600 nm of 0.6. Cells were harvested by centrifuging at 13,200 rpm (16,100 × *g*) for 5 min. The pellets were washed and resuspended in sterile phosphate-buffered saline (PBS) and were adjusted to appropriate concentrations based on the optical density for inoculations.

2.2. Bacterial inoculation of chickens

Animal experiments were conducted after receiving approval from the Chonbuk National University Animal Ethics Committee (CBU 2014-1-0038) and in accordance with the guidelines of the Korean Council for Animal Care. One-day-old Female Brown Nick layer chickens were fed antibiotic-free water and given food ad libitum. The chickens ($n = 50$) were divided evenly into either control or experimental groups. The control group chickens were given sterile PBS per os. The second group was immunized with JOL919 (0.1 mL of 1×10^9 colony forming units (cfu)) on the first day of age per os. All chickens were challenged with 0.1 mL of 1×10^9 cfu of JOL1182, which was administered during the fourth week of age per os.

2.3. Flow cytometry for CD3+CD4+ and CD3+CD8+ T cells

To evaluate the effect of one-time immunization on T cell upregulation, whole blood (1 mL) samples were collected from ten chickens via jugular vein from each group seven days post-immunization as well as post-challenge. Blood samples were mixed with 3% hetastarch (Sigma Immuno Chemicals, St. Louis, MO, USA) at a 1:2 ratio and centrifuged at 65 × *g* for 10 min to allow erythrocytes to form sediment. The supernatant cells (leukocytes) were adjusted to a concentration of 1×10^6 cells/mL in PBS. One hundred microliters of 1×10^6 isolated leukocytes were incubated with mouse Anti-chicken CD3-FITC (Fluorescein isothiocyanate), mouse Anti-chicken CD4-biot (biotin labeled), mouse Anti-chicken CD8-RPE (R-phycoerythrin) (Southern Biotech, Birmingham, AL, USA) in the dark at 4 °C for 30 min. After washing with PBS, the cells were resuspended in 100 μL of PBS and again incubated with streptavidin-APC (Allophycocyanin) antibodies (Southern Biotech, Birmingham, USA) in the dark at 4 °C for 30 min. After washing, all samples were analyzed using a FACS Calibur (BD Bioscience, Heidelberg, Germany) equipped with a 15-mW, 488-nm argon ion laser. The percentage of positively stained cells was calculated using CellQuestPro 4.0.2 software (BD Bioscience, Heidelberg, Germany).

2.4. Bacterial persistence and clearance from internal organs

Liver, spleen, and cecal samples were collected from five chickens from immunized and control groups, at 3, 5, 7 and 9 days after JOL1182 challenge. These tissues were minced in buffered peptone water (BPW, Becton, Dickinson and Company, Sparks, MD, USA) for bacterial recovery. The count of colony forming units (cfu) of JOL1182 per gram of tissue was estimated by plating 10-fold dilutions of the tissue homogenate on BGA. Homogenates were inoculated on BGA agar for enumeration and incubated overnight at 37 °C. The samples that tested negative after direct plating on BGA were pre-enriched in BPW for overnight at 37 °C, followed by enrichment in Rappaport-Vassiliadis R 10 (RV) broth (Becton, Dickinson and Company, Sparks, MD, USA) broth at 42 °C for 48 h. A loop of the enrichment broth was streaked onto BGA, and, after incubation at 37 °C for 24 h, we looked for *Salmonella*-type colonies. The amount of JOL1182 in each sample was counted from direct plating on BGA plates and expressed in $\log_{10}$ cfu/g; a sample with positive recovery only after enrichment was assumed to be 1 cfu/g, while a sample still negative after enrichment was assumed to be 0 cfu/g for data analysis. PCR analysis was performed to confirm the etiology of *Salmonella*-type strains. To differentiation the vaccine strain from the challenge strain, PCR analysis was performed for at least 10 representative colonies from each sample. Primers employed were 5-CAGGAGTTCTTACAGGTAGA-3/5-CCACACTCCGCTGTAGGTGA-3 (lon) and 5-CATCATCTGCGGGTTGCAGC-3/5-GATAATTTACCGTTA-ACGAC-3 (cpxR) for genotype amplification.

2.5. TUNEL assay

Five female Brown Nick layer chickens in each group were euthanized 3, 5 and 7 days post-challenge for TUNEL assay. Spleens were removed, fixed in 10% buffered formaldehyde, paraffin embedded, and sectioned. The terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL) assay was performed on deparaffinized sections with an apoptosis detection