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Research paper

Attenuated *Salmonella* Gallinarum secreting an *Escherichia coli* heat-labile enterotoxin B subunit protein as an adjuvant for oral vaccination against fowl typhoid

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ABSTRACT

In our previous study, we constructed a vaccine candidate (JOL916) for fowl typhoid (FT). A live adjuvant *Salmonella* Gallinarum (SG) strain was generated in the present study to facilitate efficacious oral vaccination with this vaccine. The *Escherichia coli eltB* gene secreting heat-labile enterotoxin B subunit (LTB) was cloned into an Asd$^+$ plasmid pJHL65. This was transformed into a Δ*lon* Δ*cpxR* Δ*asd* SG strain and the resulting strain was designated JOL1229. Secretion of LTB from JOL1229 was confirmed with an immunoblot assay. To determine the optimal dose of the strain, 50 six-week-old female chickens were divided into five groups (Groups A–E, $n = 10$ per group) and orally inoculated with various doses of JOL1229 and JOL916. In Group B (consisting of four parts JOL916 and one part JOL1229), significant cell-mediated immune responses, plasma IgG levels and intestinal secretary IgA levels were induced after inoculation with both strains. On challenge with the wild-type strain, significant reductions in mortality were observed in the group. In addition, after inoculation the LTB strain was not recovered in feces samples, and resulted in no, or very mild, gross lesions in the liver and spleen. Both CD4$^+$ and CD8$^+$ T-cells were significantly increased in peripheral blood samples from the chickens immunized with the LTB strain. Expression of the interleukin-6 (IL-6) gene in splenocytes was induced in the chickens immunized with the LTB strain. These results suggest that oral immunization with the LTB-adjuvant strain, in particular with the four parts JOL916 and one part JOL1229 mixture, increased the immune response and provided efficient protection against FT in chickens.

1. Introduction

Salmonella enterica serovar Gallinarum biovar Gallinarum (*Salmonella* Gallinarum, SG) causes fowl typhoid (FT), a severe septicemic disease of domesticated birds and other avian species (Pomeroy and Nagaraja, 1991). FT causes considerable economic losses in many countries throughout Latin America, South America, Africa and Asia (Shivaprasad, 2000). Various live attenuated and inactivated *Salmonella* strains have been tested as potential vaccines for FT in fowl model systems (Barrow et al., 1990; Zhang-Barber et al., 1999). Undefined mutant rough strain SG9R, one representative vaccine strain among these potential vaccine strains, was introduced in the 1950s (Smith, 1956) and was recommended for control against FT (Lee et al., 2005). However, SG9R leads to hepatitis and splenic lesions due to some residual virulence (Silva et al., 1981) and does not provide sufficient protection against wild-type SG infection (Bouzoubaa et al., 1989). In particular, the biggest disadvantage of SG9R vaccination is the fact that it must be administered either subcutaneously or intramuscularly, which can be uneconomical and

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laborious. Recently, SG mutant (JOL916) was constructed by deleting the *lon* and *cpxR* genes, which are related to the host-pathogen interaction as a live vaccine candidate for FT (Matsuda et al., 2010a). JOL916 was demonstrated to be safe, induced the development of acquired immunity, and offered protection against FT in chickens (Matsuda et al., 2010b, 2011a,b).

It is generally accepted that cell-mediated immunity is more important than humoral immune responses for protection against *Salmonella* in poultry, while IgA antibody responses seem to play a significant role in the intestinal clearance of *Salmonella* (Barrow, 2007; Chappell et al., 2009; Van Immerseel et al., 2005). Cell-mediated immunity is crucial to controlling and clearing facultative intracellular bacteria such as *Salmonella* (Alvarez et al., 2003). Mucosal immunity is also important in protecting mucosal surfaces against invasion and colonization by microbes, blocking the uptake of ungraded antigens, including foreign proteins from ingested food and symbiotic microorganisms, and preventing the development of potentially injurious immune responses to these antigens (Holmgren and Czerkinsky, 2005). Mucosally administered vaccines have several potential advantages, including enhancement of the mucosal surfaces, as well as systemic immunity, increased stability, extended shelf life, and the lack of a need for needles or specialists to administer them (Freytag and Clements, 2005). Most antigens, on the other hand, are poor immunogens when administered mucosally, including via the oral route, and at worst have results in terms of immunological tolerance or unresponsiveness as a result of induced tolerance (Yuki and Kiyono, 2003). It is well-known that *Escherichia coli* heat-labile enterotoxin B subunit protein (LTB) is robust as an adjuvant and is capable of modulating immune responses (Fischer et al., 2010). LTB also has the potential to act as an efficient carrier without danger of toxification after administration (Fingerut et al., 2005).

In this study, an attenuated SG strain containing a recombinant plasmid harboring the *eltB* gene encoding the LTB protein was constructed as a live adjuvant for oral vaccination to prevent FT in chickens. We evaluated the adjuvant effect of this LTB strain by observing the immune responses and protection efficacy, and also determined the optimal dose of the strain for oral inoculation. In addition, the safety of the strain was analyzed by evaluating fecal shedding after inoculation, and the presence of gross lesions and bacterial recovery from the liver and spleen in immunized chickens. The T-lymphocyte subpopulations from peripheral blood samples and interleukin-6 (IL-6) mRNA expression in splenocytes were also evaluated to provide more specific immunological data.

2. Materials and methods

2.1. Experimental animals

Six-week-old female Lohmann Brown layer chickens were used in the study. All birds were given ad libitum access to water and antibiotic-free feed. The animal experiments in this study were performed under the approval of the Chonbuk National University Animal Ethics Committee in accordance with the guidelines of the Korean Council on Animal Care (CBU 2011-0017).

2.2. Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are listed in Table 1. The vaccine candidate strain, JOL916, was constructed by deleting the *lon* and *cpxR* genes of the wild-type SG strain, JOL394 (Matsuda et al., 2010a). The SG strain JOL967 was constructed by deleting the *asd* gene of JOL916 using an allelic exchange method (Kang et al., 2002) and was used as the delivery strain for the *eltB* gene encoding the LTB protein. The wild-type JOL420 SG strain was used as the challenge strain. An Asd^+ plasmid, pJHL65, was used for delivering the *eltB* gene. All bacterial strains in this study were grown at 37 °C in Luria-Bertani (LB) broth (Becton, Dickinson and Company, Sparks, MD, USA). Diaminopimelic acid (DAP) (Sigma, St. Louis, MO, USA) was added (50 μg/ml) to facilitate the growth of the JOL967 strain.

2.3. Cloning the LTB gene

For construction of the LTB strain, a DNA fragment of the *eltB* gene from LTB^+ *E. coli* was amplified using eltB-F and eltB-R primers (Table 2). Amplified DNA digested with *Eco*RI and *Hin*dIII was cloned into pJHL65 digested with the same restriction enzymes, thereby resulting in pJHL65-LTB (Fig. 1). The pJHL65-LTB plasmid was transformed into JOL967 by electroporation and the resultant LTB adjuvant strain was designated as JOL1229.

2.4. Immunoblot analysis

LTB protein secretion from the JOL1229 strain was verified using immunoblot analysis. The strain was cultured in LB broth at 37 °C with continuous shaking in order to prepare the secreted proteins from JOL1229. The culture was harvested by centrifugation at $3400 \times g$ for 20 min at an optical density (OD) at 600 nm (OD_{600}) of 0.8. The cultured supernatants were passed through a 0.22 μm pore size filter and precipitated overnight with 20% (v/v) trichloroacetic acid (TCA) to detect secreted forms of proteins. The protein samples from the JOL1229 strain were boiled for 5 min at 95 °C and separated by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Subsequent immunoblot analysis was performed as previously described (Hur and Lee, 2011).

2.5. Immunization of the chickens

In experiment 1, 50 six-week-old female chickens were divided into five groups ($n = 10$ per group) to determine the optimal immunization dose for the LTB adjuvant strain. Each bird in the immunized groups was orally inoculated with 200 μl of bacterial suspension containing 1×10^8 colony-forming units (cfu), and each chicken in the control group was given 200 μl of PBS. Group A chickens were immunized with a mixture consisting of one part JOL916 (0.5×10^8 cfu) and one part JOL1229 (0.5×10^8 cfu). Group B chickens were immunized with a