J. Microbiol. Biotechnol. (2005), **15**(3), 609–615

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

© The Korean Society for Microbiology and Biotechnology

Safety and Immunogenicity of *Salmonella enterica* Serovar Typhimurium *llaB* in Mice

CHO, SUN-A, IN-SOO LEE[1], JONG-HWAN PARK, SEUNG-HYEOK SEOK, HUI-YOUNG LEE, DONG-JAE KIM, MIN-WON BACK, SEOK-HO LEE[2], SOOK-JIN HUR[2], SANG-JA BAN[2], YOO-KYOUNG LEE[2], AND JAE-HAK PARK*

Department of Laboratory Animal Medicine, College of Veterinary Medicine and School of Agricultural Biotechnology, Seoul National University, San 56-1, Sillim-Dong, Kwanak-Gu, Seoul 151-742, Korea
[1]Department of Microbiology, Hannam University, 133 Ojeong-dong, Daedeok-gu, Daejon 306-791, Korea
[2]Korean Food and Drug Administration, 5 Nokbun-dong, Eunpyung-Gu, Seoul 122-704, Korea

Received: October 6, 2004
Accepted: February 4, 2005

Abstract The safety and immunogenicity of an attenuated recombinant *Salmonella* vaccine strain, *Salmonella enterica* serovar Typhimurium *llaB*, was assessed. This vaccine strain could survive in low pH condition, and its ability of intracellular survival did not differ from that of *S. enterica* serovar Typhimurium UK1, which is the wild-type of the vaccine strain. The mortality of the mice orally administered with the vaccine strain was 50% at the dose of 10^7 CFU. All mice administered with 10^5 or 10^3 CFU of the vaccine strain survived for 3 days postinoculation (pi). However, all mice administered with more than 10^3 CFU of the vaccine strain died within 3 days pi. To examine the protective effect of the vaccine strain, mice were orally immunized with 10^4 and 10^6 CFU of the bacteria. Control mice were given with 0.5 ml of phosphate buffered saline (PBS). After 8 days, the mice were challenged with 10^9 CFU of *S. enterica* serovar Typhimurium UK1, and mortality was examined for 5 days. The survival rates of the mice immunized with 10^4 and 10^6 CFU of the vaccine strain were 60% and 80%, respectively, whereas all control mice died within 2 days after challenging. To investigate the immunogenicity of *S. enterica* serovar Typhimurium *llaB*, mice were orally immunized with 10^5 or 10^6 CFU ml of the vaccine strain. Five mice of each group were sacrificed at 5 and 12 days after immunization, and results showed that immunization of the vaccine strain led to increases of IgG1, IgG2, and IgM titers against *S. enterica* serovar Typhimurium UK1 in mouse sera, cytokine expressions such as IL-2, IL-4, IL-6, and IL-10 in spleen, and the lymphocyte proliferation response to mitogens (concanavalin A or LPS) stimulation.

*Corresponding author
Phone: 82-2-880-1256; Fax: 82-;
E-mail: pjhak@snu.ac.kr

Key words: Attenuated recombinant *Salmonella* vaccine, *S. enterica* serovar Typhimurium *llaB*, safety, immunogenicity

According to the World Health Organization (WHO), about 16 million cases of typhoid fever have been reported annually, and close to 600,000 deaths occur in areas of endemicity in Africa and Asia [11, 16, 17]. Chloramphenicol and ampicillin have been effectively used for many years, but treatment and control of typhoid fever have become increasingly difficult, because strains of *S. enterica* serovar Typhi resistant to these antibiotics have emerged worldwide [19].

Live oral typhoid vaccine Ty21a is one of the safest and best tolerated vaccines of all licensed vaccines [3, 24]. However, the modest immunogenicity of this vaccine, which requires three or four spaced doses (with an interval of 48 h between doses) to confer the credible protection, constitutes an important and practical shortcoming [3, 6, 8, 9, 13, 21]. These shortcomings stimulated efforts to identify alternative attenuated *S. enterica* serovar Typhi and *S. enterica* serovar Typhimurium strains that are well tolerated as Ty21a but much more immunogenic, so that they can be administered as single-dose live oral vaccines. *S. enterica* serovar Typhimurium is a Gram-negative facultative intracellular bacterium that causes systemic infection in mice, similar to human typhoid fever [1, 16]. Therefore, *S. enterica* serovar Typhimurium has been considered as a suitable strain to study human typhoid fever and as a candidate vector in vaccine design [23]. Recently, attenuated *Salmonella* spp. gained attention, because they can be administered mucosally and stimulate humoral and cell-mediated immune

response [16, 18–20, 22]. A variety of attenuated *Salmonella* strains such as CDV 908, CDV 906, CDV 906-*htrA*, *phoP/phoQ*, and *rpoS/R* mutants have been used as live vaccines to induce mucosal as well as systemic immunity against either carrier itself or heterologous antigen expressed [6, 8, 13, 22, 24]. Recombinant *Salmonella* vaccines have also been developed as multivalent vaccines to deliver recombinant antigens originating from viruses, bacteria, and parasites [24]. Also, live attenuated *Salmonella* strains that express a foreign antigen can be administered via the oral route and can induce strong mucosal and systemic immune response, to the foreign antigen, conferring protective immunity against numerous pathogens in several animal models. Despite encouraging preclinical results, however, a few clinical trials with live recombinant *Salmonella* stains showed weak to undetectable human immune response to the foreign antigens, indicating the need for further optimization [19]. We developed an attenuated recombinant *Salmonella* vaccine strain, *S. enterica* serovar Typhimurium *llaB*. In the present study, the safety and immunogenicity of this vaccine candidate in mice were evaluated.

MATERIALS AND METHODS

Mice

Four-week-old specific pathogen-free BALB/c male mice (Orient, Seoul, Korea) were used in this study. They were housed in individual cages at 22±2°C under a 14/10 h light/dark cycle. Food and water were provided *ad libitum*. All animal experiments were performed in accordance with the laboratory animal guidelines of Seoul National University.

Bacterial Strain

Bacteria were cultured at semi-aerobic conditions in 3 ml of medium in 13×100 mm test tubes with shaking of 240 rpm at 37°C. The bacterial strain used in this study was *S. enterica* serovar Typhimurium *llaB::MudJ*. Acid-sensitive mutant, *S. enterica* serovar Typhimurium *llaB*, was isolated using P22-mediated MudJ (Km, *lacZ*) transduction of a wild-type *S. enterica* serovar Typhimurium UK1, as described by Holly and Foster [7]. About 20,000 transductants were patched onto LB plates with kanamycin (50 μg/ml) and grown overnight at 37°C. Grown patches were replicated to LB plates containing dinitrophenol (200 μM) and incubated at 37°C. Survivors were rescued after 5 h by replicating onto LB plates [4]. Expected mutants that did not survive under this treatment were restricted from the original master plates, and named *S. enterica* serovar Typhimurium *llaB*.

Acid Tolerance Response

To examine the pH tolerance of *S. enterica* serovar Typhimurium *llaB* and *S. enterica* serovar Typhimurium UK1, the pH of LB broth was adjusted to 3.0, 5.0, 6.0, and 7.2 (control) by addition of NaOH. Two-hundred μl of overnight-cultured *S. enterica* serovar Typhimurium *llaB* and *S. enterica* serovar Typhimurium UK1 were added to each tube containing 10 ml each of pH-adjusted LB broth and incubated at 37°C. OD at 600 nm was measured at 0, 0.5, 1, 2, 4, 5, and 6 h post-incubation at 37°C with shaking of 220 rpm. At that time, the viable bacteria were counted by plating onto LB agar. This process was performed twice.

Invasion and Intracellular Survival Assay

HT-29 human colon adenocarcinoma cells (Korean Cell Line Bank: KCLB, Seoul, Korea) were cultured and maintained in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum (Gibco, Grand Island, NY, U.S.A.) and 1 mM penicillin-streptomycin (Sigma-Aldrich, St. Louis, MO, U.S.A.). The cells were grown at 37°C in a 5% CO_2 atmosphere. Prior to invasion assays, cells were passaged several times in the absence of antibiotic. One ml of RPMI-1640 medium containing 4×10^5 cells was added into each well of a 24-well tissue culture plate and incubated overnight. Bacteria were incubated overnight in RPMI-1640 medium without antibiotics, and the number of bacteria was estimated by measuring the OD of the culture at 600 nm. Approximately 10^7 CFU of the bacteria were added to HT-29 cells and incubated for 4, 24, 48, and 72 h. Cells were subsequently washed three times with phosphate buffered saline (PBS; pH 7.2), and the growth of extracelluar bacteria was inhibited by replacing the medium with RPMI medium containing 50 μg of ampicillin per ml. Cells were further incubated for 1 h, washed three times with PBS, and lysed by addition of 1 ml of 1% Triton X-100 (Sigma, U.S.A.) in PBS for 30 min at 37°C. The plates were shaken vigorously for 5 min to ensure lysis. The lysates were serially diluted ten-fold in PBS and plated onto LB agar.

Virulence and Efficacy of *S. enterica* Serovar Typhimurium *llaB*

To examine the virulence of *S. enterica* serovar Typhimurium *llaB*, overnight-incubated *S. enterica* serovar Typhimurium *llaB* were diluted with PBS to make concentrations of 10^9, 10^7, 10^5, and 10^3 CFU/0.5 ml. Ten mice of each group were orally administered with *S. enterica* serovar Typhimurium *llaB*, and mortality was then examined for three days. To examine the efficacy of *S. enterica* serovar Typhimurium *llaB*, five mice of each group were orally administered with 10^4 and 10^6 CFU/0.5 ml of *S. enterica* serovar Typhimurium *llaB*. At 8 days after immunization, mice were orally challenged with 10^9 CFU/0.5 ml of *S. enterica* serovar Typhimurium UK1, and mortality was examined for five days.

Immunogenicity of *S. enterica* Serovar Typhimurium *llaB*

Mice of each group were orally immunized with 10^5 and 10^6 CFU/0.5 ml of *S. enterica* serovar Typhimurium *llaB*

grown, respectively, while the control mice were administered with 0.5 ml of PBS. At 5 and 12 days after immunization, all mice were anesthetized with ether, and blood samples were collected by a cardiac puncture using a heparin-treated syringe. The mice were then necropsied, and spleens were sampled.

Proliferation Assay of T- and B-Cells in Spleen. The spleens were removed aseptically from mice and placed individually into Petri dishes containing 3 ml of complete RPMI-1640 medium. As previously described [2, 12], single-cell suspensions were prepared by chopping the spleens into small pieces with sterile scissors and then forcing the spleen tissue up and down through a 3 ml syringe. The suspension was transferred to a 15 ml conical tube containing 3 ml of complete RPMI-1640 and centrifuged at 1,700 rpm for 10 min. The cell pellet was resuspended in ACK lysis buffer (Tris-NH_4Cl) and incubated for 15 min with occasional mixing to lyze erythrocytes. After washing twice in complete RPMI-1640, the suspensions were adjusted to a final concentration of 2×10^6 cells/ml in complete RPMI-1640. Proliferation responses of spleen cells to mitogens were determined using a commercial cell proliferation ELISA kit (Roche Diagnostics, Mannheim, Germany). Briefly, 10^5 cells in 50 µl of complete RPMI-1640 medium were added to the wells of a 96-well tissue culture plate and cultured in the presence or absence of T- and B-cell mitogens. Fifty µl of concanavalin A (Con A, 2.5 µg/ml) and lipopolysaccharide (LPS, 5 µg/ml) were added to the wells. Control wells received 50 µl of complete medium. The cells were cultured for 72 h at 37°C in a humidified incubator supplemented with 5% CO_2. Cell proliferation during the final 18 h of culture was determined by measuring the incorporation of 5-bromo-2'-deoxyuridine (BrdU) using peroxidase-conjugated anti-BrdU antibodies and a peroxidase substrate system. The absorbance of each well was read at 450 nm using an ELISA reader (Sunrise, TECAN Austria GmbH, Salzburg, Austria).

Estimation of Antibody Responses. An ELISA was used to examine the antibody level to *S. enterica* serovar Typhimurium UK1. *S. enterica* serovar Typhimurium UK1 was cultured in tryptic soy broth and collected. The bacteria were washed three times with sterilized PBS and broken with a sonicator. Twenty-five µg of total bacterial protein were loaded into each well of a 96-well ELISA plate and incubated overnight at 4°C. After washing three times with PBS, the plate was blocked with 1% bovine serum albumin at 4°C for 2 h and incubated with sera (1:50 diluents) of mice at room temperature (RT) for 2 h. Then, it was washed three times with 0.05% Tween-PBS (T-PBS) and incubated with peroxidase-conjugated anti-mouse IgG_1, IgG_{2a}, and IgA antibodies (Zymed, San Francisco, CA, U.S.A.) at room temperature (RT) for 2 h. Finally, it was washed three times with T-PBS, visualized with *o*-phenylenediamine dihydrochloride (Sigma, U.S.A.), and absorbance was read at 450 nm.

Semi-Quantitative RT-PCR for Cytokines. A portion of spleen samples was added to 1 ml of Trizol reagent (Invitrogen, Carlsbad, CA, U.S.A.), and was electrically homogenized (Ultraturrax T8, IKA, Heidelberg, Germany) in 5-ml round-bottom glass tubes in 500 µl of Trizol on ice. Each homogenate was transferred to a 1.5-ml Eppendorf tube, vortex-mixed, and incubated at RT for 5 min. Two-hundred µl of chloroform were added, and the tubes were vortex-mixed for 15 s and placed on ice for 10 min. Phases were separated by centrifugation at 12,000 rpm for 20 min in a refrigerated microcentrifuge. The upper aqueous phase was transferred to a new 1.5 ml tube, and RNA was precipitated by the addition of 0.5 ml of isopropanol. The tubes were allowed to stand at −20°C for at least 12 h. The precipitate was pelleted by centrifugation at 12,000 rpm for 15 min at 4°C, and the pellets were washed with 1 ml of 70% ethanol. After removal of the supernatant, the pellets were dried at RT for 20 min before resuspension in 100 µl of diethyl pyrocarbonate-treated water. The RNA concentration was determined by absorbance at 260 nm. cDNA was reverse transcribed from 1 µg of total RNA using a GeneAmp RNA PCR Core kit (Applied Biosystems, Foster City, CA, U.S.A.). PCR was performed using i-*Taq* DNA polymerase (iNtRON, Sungnam, Kyungki-

Table 1. Primer sequences, annealing temperature, and the sizes of the PCR products.

Cytokines	Sequences (5'-3')	Annealing temp (°C)	Product (bp)
IL-2	F: CCCCATGATGCTCACGTTTA[a] R: TTCCAGGCACTGGAGATGTTT	54	73
IL-4	F: CAGAGCTATTGATGGGTCTCA[a] R: TGGTGGCTCAGTACTACGAGT	54	446
IL-6	F: CATGTTCTCTGGGAAATCGTGG[a] R: AACTGATATGCTTAGGCATAACGCAC	52	492
IL-10	F: AGCTGGACAACATACTGCTAACCG[a] R: TTTTCCAAGGAGTTGTTTCCGTTAG	52	461
β-actin	F: ATGGATGACGATATCGCT[b] R: ATGAGGTAGTCTGTCAGGT	56	569

[a]Designed by the primer selection program of BCM Search Launcher (Baylor College of Medicine, Human Genome Sequencing Center, Houston, TX, U.S.A.) and used for sequencing of the 16S rRNA gene.
[b]From reference 9.

Do, Korea) and specific primer sets for cytokines. The sequences of the primers for cytokines, annealing temperature, and sizes of PCR products are shown in Table 1. PCR conditions were as follows: 3 min of denaturation at 95°C, followed by 35 cycles consisting of 45 sec of denaturation at 94°C, 45 sec of annealing at 52–54°C, and 1 min of extension at 72°C, and 5 min of final extension at 72°C after amplification. The PCR products were resolved on 1.5% agarose gel with ethidium bromide and analyzed using a model Gel-Doc 2000-Quantity one Discovery Series™ (BIO-RAD Laboratories, Inc., Hercules, CA, U.S.A.) and Kodak Digital Science 1D-Scientific Imaging System software (Kodak, New Haven, CT, U.S.A.). The scanned images were digitized and quantified using Kodak Digital Science 1D analysis software, and the values for each cytokine were compared to those of β-actin. Each sample in all experiments was analyzed in duplicate.

Statistical Analysis

Significant differences between the experimental and control groups were determined using Duncan's Multiple Range Test (SAS ver. 8.1, SAS Institute Inc., Cary, NC, U.S.A.). Values of $p<0.05$ were considered significant.

Results

Acid Tolerance and Intracellular Survival Assay

The pH tolerance of *S. enterica* serovar Typhimurium UK1 and *S. enterica* serovar Typhimurium *llaB* were evaluated (Fig. 1). Both bacteria incubated in pH 7.2 or 6.0-adjusted medium grew continuously; however, the growth curve of *S. enterica* serovar Typhimurium UK1 incubated in pH 3.0 or 5.0-adjusted medium decreased after 30 min. On the other hand, *S. enterica* serovar Typhimurium *llaB* incubated in pH 3.0 or 5.0-adjusted medium showed growth pattern similar to that incubated in pH 7.2 or 6.0-adjusted medium. In the intracellular survival assay using HT-29 cell, the number of both intracellular bacteria decreased with time. *S. enterica* serovar Typhimurium *llaB* strain showed a significantly lower intracellular survival rate than that of *S. enterica* serovar Typhimurium UK1 at 2, 48, and 72 h (Fig. 2).

Virulence and Efficacy of *S. enterica* Serovar Typhimurium *llaB*

The virulence of *S. enterica* serovar Typhimurium UK1 and *S. enterica* serovar Typhimurium *llaB* in mice was evaluated. All the mice administered with 10^3 CFU or more of *S. enterica* serovar Typhimurium UK1 died. All the mice administered with 10^9 CFU of *S. enterica* serovar Typhimurium *llaB* died within 3 days pi; however, the mice administered with 10^7 CFU of the bacteria showed 50% mortality (Table 2). None of the mice administered with 10^3 or 10^5 CFU of *S. enterica* serovar Typhimurium *llaB* died (Table 2). Based on these results, 10^4 and 10^6 CFU of

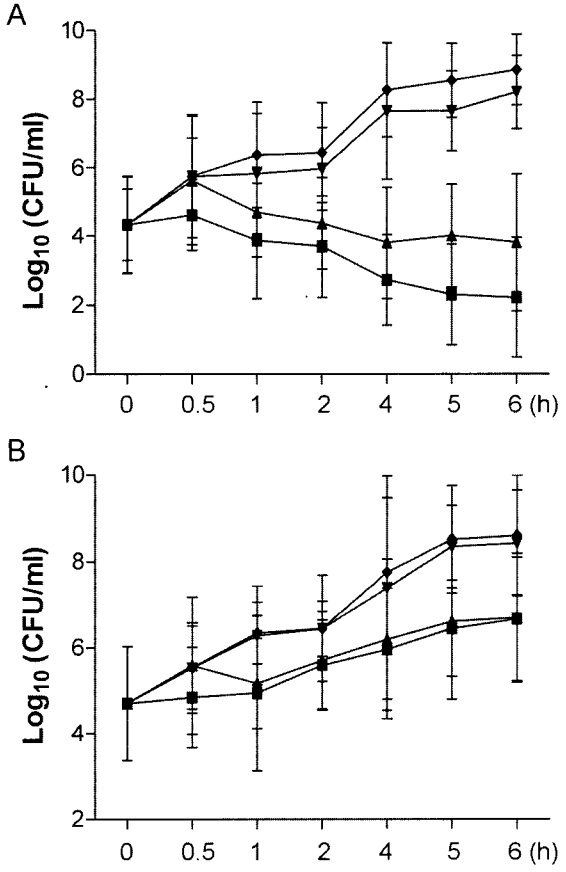

Fig. 1. Acid tolerance response of *S. enterica* serovar Typhimurium UK1 (A) and *S. enterica* serovar Typhimurium *llaB* (B).
Results are expressed as mean±SD. Each symbol indicates pH 3.0 (-■-), 5.0 (-▲-), 6.0 (-▼-), and 7.3 (-◆-).

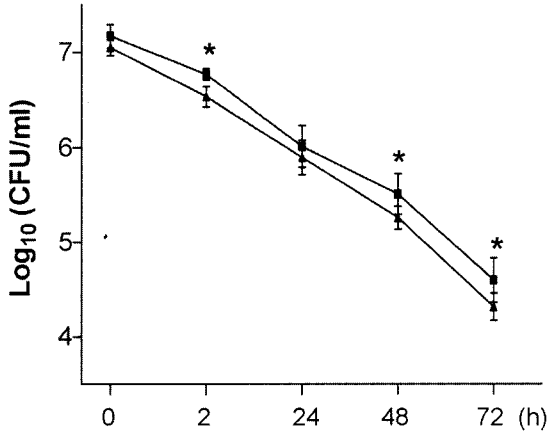

Fig. 2. Intracellular survival of *S. enterica* serovar Typhimurium UK1 (-■-) and *S. enterica* serovar Typhimurium *llaB* (-▲-) in intestinal epithelial cell line HT-29.
Results are expressed as mean±SD.

Table 2. The virulence of *S. enterica* serovar Typhimurium UK1 and *S. enterica* serovar Typhimurium *llaB*.

Inocula dose (CFU)	[a]Survival no./Total no.	
	S. enterica serovar Typhimurium UK1	*S. enterica* serovar Typhimurium *llaB*
10^3	0/10	10/10
10^5	0/10	10/10
10^6	ND[b]	10/10
10^7	0/10	5/10
10^9	0/10	0/10

[a]Mortality was examined for three days.
[b]Not determined.

S. enterica serovar Typhimurium *llaB* were determined to be the immunizing dose for the efficacy test. When challenged with 10^9 CFU of *S. enterica* serovar Typhimurium UK1, the survival rate of the mice immunized with 10^4 or 10^6 CFU of *S. enterica* serovar Typhimurium *llaB* were 60 and 80%, respectively (Fig. 3). However, all control mice administered with PBS instead of *S. enterica* serovar Typhimurium *llaB* died within 2 days after challenging with *S. enterica* serovar Typhimurium UK1.

Proliferation Assay

The proliferative responses of spleen cells isolated from the mice administered with 10^5 and 10^6 CFU of *S. enterica* serovar Typhimurium *llaB* to stimulation with ConA were significantly higher than those from the control mice at 5 and 12 days after immunization ($p<0.05$, Fig. 4). The proliferation of B-cells in response to stimulation with LPS in the spleen of the mice administered with 10^5 or 10^6 CFU of *S. enterica* serovar Typhimurium *llaB* was significantly higher than in that of control mice at 5 days after immunization.

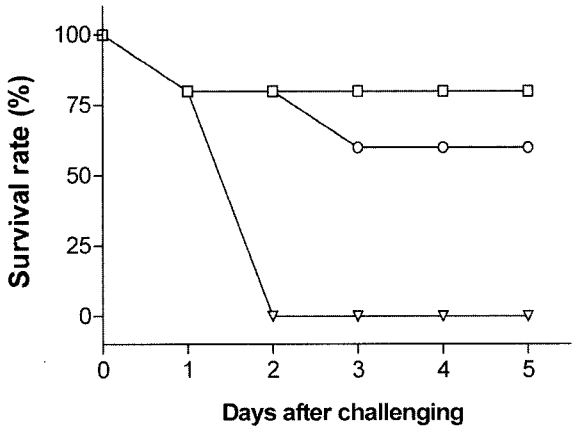

Fig. 3. The survival rate of mice after challenging with *S. enterica* serovar Typhimurium UK1.
Mice of each group were orally immunized with 10^4 (-△-) or 10^6 CFU (-□-) of *S. enterica* serovar Typhimurium *llaB*. Control mice (-▽-) received 0.5 ml of PBS.

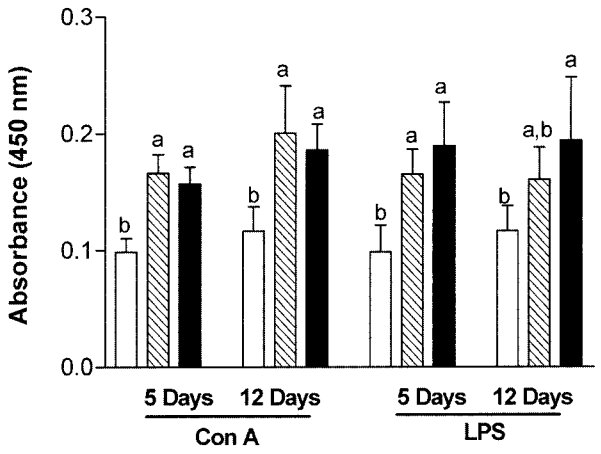

Fig. 4. Proliferation of spleen cell in response to stimulation of Con A or LPS at 5 and 12 days after immunization.
Mice of each group were orally immunized with 10^5 (the bar with the oblique line) or 10^6 (the dark bar) CFU of *S. enterica* serovar Typhimurium *llaB*. Control mice (the white bar) received 0.5 ml of PBS. Results are expressed as mean±SD. [a,b]Means with the same letter are not significantly different ($p< 0.05$).

However, the B-cells proliferation was significantly higher in the spleen of 10^6 CFU-immunized mice, but not 10^5 CFU-immunized mice, than in that of control mice at 12 days after immunization ($p<0.05$, Fig. 4).

Estimation of Antibody Responses

Five days after immunization, titers of all tested immunoglobulins such as IgG_1, IgG_{2a}, IgA, and IgM were significantly higher in sera of the mice administered with 10^5 and 10^6 CFU of *S. enterica* serovar Typhimurium *llaB* than in those of control mice, except that of IgG_{2a} in sera of the mice with 10^5 CFU of the bacteria ($p<0.05$, Table 3). At 12 days after immunization, sera IgG_1, IgG_{2a}, IgA, and IgM titers of all the mice administered with 10^5 and 10^6 CFU of *S. enterica* serovar Typhimurium *llaB* were significantly higher than those of control mice ($p<0.05$, Table 3). Except for sera IgM, the titers of all immunoglobulin types at 12 days after immunization were higher than those at 5 days after immunization. There was no significant difference in antibody titer between the groups administered with 10^5 and 10^6 CFU of *S. enterica* serovar Typhimurium *llaB* ($p<0.05$).

Semi-Quantitative RT-PCR for Cytokines

mRNA expressions of IL-2, IL-4, IL-6, and IL-10 were significantly higher in the spleens of the mice administered with *S. enterica* serovar Typhimurium *llaB* than in those of control mice. The expression of all cytokines in the spleen was dose dependent; however, they did not show any significant difference between the groups administered with 10^5 and 10^6 CFU of *S. enterica* serovar Typhimurium *llaB* ($p<0.05$, Table 4). The expression levels of all cytokines

Table 3. ELISA for detection of the levels of IgG_1, IgG_{2a}, IgA, and IgM from sera of mice immunized with 10^5 or 10^6 CFU of *S. enterica* serovar Typhimurium *llaB* at 5 and 12 days after immunization.

	IgG_1	IgG_{2a}	IgA	IgM
5 days				
Control	0.087±0.008[b]	0.085±0.003[b]	0.104±0.005[b]	0.118±0.003[b]
llaB 10^5 CFU	0.169±0.023[a]	0.092±0.009[a,b]	0.142±0.048[a]	0.348±0.021[a]
llaB 10^6 CFU	0.172±0.031[a]	0.102±0.015[a]	0.139±0.037[a]	0.376±0.031[a]
12 days				
Control	0.125±0.013[b]	0.072±0.008[b]	0.115±0.006[b]	0.160±0.021[b]
llaB 10^5 CFU	0.266±0.069[a]	0.158±0.032[a]	0.221±0.041[a]	0.331±0.035[a]
llaB 10^6 CFU	0.252±0.014[a]	0.169±0.037[a]	0.184±0.045[a]	0.364±0.011[a]

[a,b]Means with the same letter are not significantly different ($p<0.05$).

were not significantly different at between 5 and 12 days after immunization (Table 4).

DISCUSSION

This study was performed to examine the safety and immunogenicity of attenuated *S. enterica* serovar Typhimurium *llaB*. The results showed that the virulence of *S. enterica* serovar Typhimurium *llaB* was effectively attenuated and had the protective effect against *S. enterica* serovar Typhimurium UK1, the challenging organism. Immunization with *S. enterica* serovar Typhimurium *llaB* elicited a higher immune response, including the increase of T- and B-cell proliferation in response to stimulation of mitogens (ConA and LPS), cytokine expression such as IL-2, -4, -6, and -10 in spleen, and serum antibody titer of IgG_1, IgG_{2a}, IgA, and IgM responding to the antigen of *S. enterica* serovar Typhimurium UK1.

Lymphocyte proliferation responses to mitogens are widely used to assess T- and B-cell functions [5, 14, 15]. Mice administered with an external antigen exhibited enhanced T- and B- cell functions, as indicated by elevated proliferation responses to the T-cell mitogen, Con A, and the B-cell mitogen, LPS. In the present study, spleen cells stimulated by Con A and LPS were more proliferating in the mice immunized with *S. enterica* serovar Typhimurium *llaB* than in the control mice, indicating that *S. enterica* serovar Typhimurium *llaB* increased both T- and B-cell functions.

T-cells are the main effectors and regulators of cell-mediated immunity [5]. On activation by antigen or pathogen, T-cells synthesize and secrete a variety of cytokines that serve as growth, differentiation, and activation factors for other immunocompetent cells. Recent studies have shown that T-cells can be divided into two functional types, Th1 and Th2, based on their cytokine profile [2, 4]. Th1 cells secrete IFN-γ, IL-2, and TNF-β, whereas Th2 cells produce IL-4, IL-5, IL-6, and IL-10 [14]. Th1 cells are efficient inducers of cell-mediated immunity, including activation of macrophage and CTL, while Th2 cells provide better help for B-cell responses, including those of IgG1, IgE, and IgA isotypes. Generally, *S. enterica* serovar Typhimurium has been shown to induce a Th1-dependent immune response [14]. Both Th1 and Th2 cells may regulate IgA expression, and the Th2 cell subset has been shown to be more efficient. In the present study, RT-PCR analysis showed that administration of *S. enterica* serovar Typhimurium *llaB* to mice led to significant increase of IL-2, IL-4, IL-6, and IL-10 mRNA expressions in the spleens. These results mean

Table 4. Semi-quantitative RT-PCR analysis for IL-2, IL-4, IL-6, and IL-10 from spleen samples of mice immunized with 10^5 or 10^6 CFU of *S. enterica* serovar Typhimurium *llaB* at 5 and 12 days after immunization.

	IL-2	IL-4	IL-6	IL-10
5 days				
Control	0.929±0.086[b]	0.951±0.077[b]	0.849±0.068[b]	0.844±0.087[b]
llaB 10^5 CFU	1.218±0.096[a]	1.209±0.082[a]	1.114±0.097[a]	0.141±0.088[a]
llaB 10^6 CFU	1.375±0.097[a]	1.326±0.085[a]	1.239±0.075[a]	1.218±0.071[a]
12 days				
Control	0.859±0.109[b]	0.838±0.107[b]	0.730±0.090[b]	0.708±0.082[b]
llaB 10^5 CFU	1.058±0.096[a]	1.169±0.104[a]	1.123±0.105[a]	1.302±0.107[a]
llaB 10^6 CFU	1.281±0.097[a]	1.197±0.074[a]	1.303±0.092[a]	1.442±0.129[a]

Results were expressed as the density values of each cytokine divided with those of β-actin.
[a,b]Means with the same letter are not significantly different ($p<0.05$).

that that *S. enterica* serovar Typhimurium *llaB* induced both Th1 and Th2 immune response in mice.

In conclusion, *S. enterica* serovar Typhimurium *llaB* is an effectively attenuated strain originating from *S. enterica* serovar Typhimurium UK1 and has prominent immunogenicity. Therefore, this strain can be used as an attenuated live oral vaccine against *Salmonella* infection.

REFERENCES

1. Charfield, S., M. Roberts., P. London, I. Cropley, G. Douce, and G. Dougan. 1993. The development of oral vaccines based on live attenuated *Salmonella* strain. *FEMS Immunol. Med. Microbiol.* **7:** 1–7.
2. Cookson, B. T. and M. J. Bevan. 1997. Identification of a natural T cell epitope presented by *Salmonella* infected macrophages and recognized by T cells from orally immunized mice. *J. Immunol.* **158:** 4310–4319.
3. Ferreccio, C., M. M. Levine, H. Rodriguez, and R. Contreras. 1989. Comparative efficacy of two, three, or four doses of Ty21a live oral typhoid vaccine in enteric-coated capsule; a field trial in an endemic area. *Infect. Immun.* **159:** 766–769.
4. Foster, J. W. and B. Bearson. 1994. Acid-sensitive mutants of *Salmonella typhimurium* identification through a dinitrophenol lethal screening strategg. *J. Bacteriol.* **176:** 2596–2602.
5. Gill, H. S., D. L. Watson, and M. R. Brandon. 1992. *In vivo* inhibition by monoclonal antibody to CD4+ T cells of humoral and cellular immunity in sheep. *Immunology* **77:** 38–42.
6. Hohmann, E. L., C. A. Oletta, K. P. Killeen, and S. I. Miller. 1996. phoP/phoQ-deleted *Salmonella typhi* (Ty800) is a safe and immunogenic single-dose typhoid fever vaccine in volunteers. *J. Infect. Dis.* **173:** 1408–1414.
7. Holly, E. A. and W. Foster. 1982. Bacteriophage P22 as a vector for Mu mutagenesis in *Salmonella typhimurium:* Isolation of nad-lac and pnu-lac gene fusion. *J. Bacteriol.* **152:** 959–962.
8. Hone, D. M., C. O. Tacket, A. M. Harris, B. Kay, G. Losonsky, and M. M. Levine. 1992. Evaluation in volunteers of a candidate live oral attenuated *S. typhi* vector vaccine. *J. Clin. Invest.* **90:** 412–420.
9. Ivanoff, B., M. M. Levine, and P. H. Lambert. 1994. Vaccination against typhoid fever: Present status. *Bull. World Health Organ.* **72:** 957–971.
10. Jonson, V. J., M. Tsunoda, and R. P. Sharma. 2000. Increased production of proinflammatory cytokines by murine macrophage following oral exposure to sodium selenite but not to seleno-L-methionine. *Arch. Environ. Contam. Toxicol.* **39:** 243–250.
11. Kim, S. H., O. Y. Lim, S. H. Kim, J. Y. Kim, Y. H. Kang, and B. K. Lee. 2003. Pulsed-field gel electrophoresis and mutation typing of gyrA gene of quinolone-resistant *Salmonella enterica* serovar paratyphi a isolated from outbreak and sporadic cases, 1998–2002, Korea. *J. Microbiol. Biotechnol.* **13:** 155–158.
12. Lee, H. Y., J. H. Park, S. H. Seok, S. A. Cho, M. W. Baek, D. J. Kim, Y. H. Lee, and J. H. Park. 2004. Dietary intake of various lactic acid bacteria suppresses Type 2 helper T cell production in antigen-primed mice splenocyte. *J. Microbiol. Biotechnol.* **14:** 167–170.
13. Levine, M. M., D. Herrington, J. R. Murphy, J. G. Morris, G. Losonsky, B. Tall, A. A. Lindberg, S. Svenson, S. Baqar, and M. F. Edwards. 1987. Safety, infectivity, immunogenicity and *in vivo* ability of two attenuated auxotrophic mutant strains of *Salmonella typhi*, 541Ty and 543Ty, as live oral vaccine in man. *J. Clin. Invest.* **79:** 888–902.
14. Mosmann, T. R. and R. L. Coffman. 1989. Th1 and Th2 cells: Different patterns of lymphokine secretion lead to different functional properties. *Annu. Rev. Immunol.* **7:** 145–173.
15. Muotiala, A. and P. H. Makela. 1990. The role of INF-r in murine *Salmonella typhimurium* infection. *Microb. Pathog.* **8:** 135–141.
16. Pang, T., M. M. Levine, B. Ivanoff, J. Wain, and B. B. Finlay. 1998. Typhoid fever - important issues still remain. *Trends Microbiol.* **6:** 131–133.
17. Raupach, B. S. and H. Kaufmann. 2001. Bacterial virulence, proinflammatory cytokines and host immunity: How to choose the appropriate *Salmonella* vaccine strain? *Microbes Infect.* **3:** 1261–1269.
18. Sebastiani, G., V. Blais, V. Sancho, S. N. Vogel, M. M. Stevenson, P. Gros, J. M. Lapointe, S. Rivest, and D. Malo. 2002. Host immune response to *Salmonella enterica* serovar typhimurium infection in mice derived from wild strains. *Infect. Immun.* **70:** 1997–2009.
19. Sirard, J. C., F. Niedergang, and J. P. Kraehenbuhl. 1999. Live attenuated *Salmonella*: A paradigm of mucosal vaccines. *Immunol. Rev.* **171:** 5–26.
20. Staats, H. F., R. J. Jackson, M. Marinaro, I. Takahashi, H. Kiyono, and J. R. McGhee. 1994. Mucosal immunity to infection with implication for vaccine development. *Curr. Opin. Immunol.* **6:** 572–583.
21. Tacket, C. O., D. M. Hone, R. I. Curtiss, S. M. Kelly, G. Losonsky, L. Guers, A. M. Harris, R. Edelman, and M. M. Levine. 1992. Comparison of the safety and immunogenicity of *aroCaroD* and *cya,crp Salmonella typhi* strains in adult volunteers. *Infect. Immun.* **60:** 536–541.
22. Tacket, C. O., M. B. Sztein, S. S. Wasserman, G. Losonsky, K. L. Kotloff, T. L. Wyant, J. P. Nataro, R. Edelman, J. Perry, P. Bedford, D. Brown, S. Chatfield, G. Dougan, and M. M. Levine. 2000. Phase 2 clinical trial of attenuated *Salmonella enterica* serovar *typhi* oral live vector vaccine CVD 908-htrA in U.S. volunteers. *Infect. Immun.* **68:** 1196–1201.
23. Thatte, J., S. Rath, and V. Bal. 1995. Analysis of immunization route-related variations in the immune response to heat-killed *Salmonella typhimurium* in mice. *Infect. Immun.* **63:** 99–103.
24. Wang, J. Y., M. F. Pasetti, F. R. Noriega, R. I. Anderson, S. S. Wasserman, J. E. Galen, M. B. Sztein, and M. M. Levine. 2001. Construction, genotyping and phenotyping characterization, and immunogenicity of attenuated Δ*guaBA Salmonella enterica* serovar typhi strain CDV 915. *Infect. Immun.* **69:** 4734–4741.