Immune Responses of BALB/c Mice Administered via Oral Route to a Combined Salmonella Typhimurium Ghost Vaccine

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Received June 15, 2015 / Revised August 27, 2015 / Accepted October 26, 2015

Salmonella Typhimurium (ST) JOL389 and χ3339 are strong virulent strains against mouse. ST χ8554 is derived by deletion of the asl gene from ST χ3339. Plasmid pMMP184 carrying a ghost cassette was transformed into ST χ8554, and ST χ8554 ghost cells were prepared and administrated via the oral route to BALB/c mice. Change in the amount of total IgG was not elicited to boosting of single ST χ8554 ghost cells, but the content was increased from 6 weeks after the 3rd administration. However, when the ST JOL389 ghost cells is administered together with ST χ8554 ghost cells, the content of total IgG was increased in 2 weeks post primary administration. It was found that the content of total IgG of the group mixed with ST JOL389 ghost cells showed an increased value of 8 times or more at 10 weeks when compared with the group of ST χ8554 ghost cells. The content of IgG1, IgG2a, and IgA in both groups increased from 4 weeks postprimary administration. As a challenge test of virulent ST χ3339, χ8554 (pMMP184) and χ8554 (pMMP184)/JOL389 ghost cell groups showed protection of 50% or more when compared to the control group. These results suggest that the preparation of combined ghost cells from a strong virulent ST increases immunity more than a single strain.

Key words : BALB/c mouse, ghost vaccine, immune, protection, S. Typhimurium, virulence

Introduction

The piglets have frequently occurred in death or suppression of promotion as a result of the spread of disease in the digestive organ. The dominant bacteria to cause gastrointestinal disease have formed a mainstream by Salmonella and pathogenic Escherichia coli (E. coli). Thus, Salmonella ghost cells carrying a pathogenic E. coli antigen have been developed as a means to protect the digestive organ from the bacterial diseases [2, 9]. A polyvalent ghost vaccine for protections against pathogenic E. coli and Salmonella is able to construct through the following process; 1) Antigen of particular interest is selected as a foreign antigen from enterotoxigenic Escherichia coli (ETEC). 2) The gene of the selected foreign antigen is cloned into a vector which is capable of expressing constitutively a large amount and can be anchored on the cell membrane. 3) The recombinant plasmid carrying the antigen gene is produced through cloning a cassette to induce formation of ghost cells in response to specific conditions including temperature (cI857-APr-E gene) [8, 21]. 4) The recombinant plasmid is transformed into Salmonella, and Salmonella ghost cells are produced by specific condition(s) such as an elevated incubation temperature. In the case of the production of ghost cell depending on temperature, a strain transformed by a plasmid harboring the ghost cassette (cI857-APr-E gene) is cultured in order to obtain a specific amount of cell mass at a temperature less than 30°C, whereas the ghost cells are formed at temperature more than 42°C [3, 9].

Since bacterial ghost is native state of bacteria in the lysis process, it is well conserved for all the elements of the major immune stimulation [15]. These factors are referred to pathogen-associated molecular patterns (PAMPs), which include lipopolysaccharide (LPS), monophosphoryl lipid A (MPL), peptidoglycan, and flagella. PAMPs elicit an innate immune response via toll like receptor (TLR), which all bacteria induced to bacterial ghost is capable of eliciting an innate immune response primarily as biological reactions [15]. In addition, bacterial ghost not only induces humoral and cellular immune responses in experimental animals, but also has a characteristic as an effective adjuvant [19].
In this study, we evaluated the immune ability by the mixed ghost cells derived from ST χ8554 and strong virulent ST JOL389. Furthermore, we examined the protective ability of the vaccinated BALB/c mice with the combined ghost cells against ST χ3339.

Materials and Methods

**Bacterial strains, plasmids, and reagents**

The bacterial strains and plasmids used for this study are listed in Tables 1. *S. Typhimurium* and *Escherichia coli* were grown at 28, 37 or 42°C in Luria-Bertani (LB) or M9 minimal medium supplemented with 1.5% agar [1]. Antibiotics were added to the culture media at the following concentrations: ampicillin, 100 μg/ml; streptomycin, 50 μg/ml. When required, L-arabinose and diaminopimellic acid (DAP, USA) was added to final concentrations of 0.2% and 50 μg/ml in the medium.

**General DNA manipulations**

DNA manipulations were done as described by Sambrook et al. [20]. Plasmids were introduced into *E. coli* competent cells via heat-shock with CaCl2 treatment and into *Salmonella* competent cells via electroporation [20].

The ghost cassette was PCR-amplified from pLDR20. Oligonucleotides used for PCR are as follows; Ghost-F-XbaI; 5′-TCTAGAGACCAGAACACCTTGCCGATC-3′ and Ghost-R-XbaI 5′-TCTAGACCTTAC A TCACCGCTCCG-3′. Taq DNA polymerase for PCR amplification was employed by EX-Tag (TaKaRa, Japan) or Eco-Tag (Solgent, South Korea), and Pfu DNA polymerase was purchased from invitrogen (USA). Recombinant plasmid carrying target gene was identified by digestion of restriction enzyme, PCR and nucleotide sequencing (Macrogen, South Korea).

**Assays of viable cell and formation of ghost cells**

The ghost cells were inoculated 1% into 100 ml fresh LB medium by grown cells carrying ghost cassette, and then grown at 28°C above absorbance 1.5 at 600 nm. The grown cells were prepared to ghost cells via shifting temperature from 28°C to 42°C[6, 7]. When the number of viable cells was reduced to 10^9 CFU/ml or less, the ghost formation was forced to shut down. The ghost cells were washed with distilled water, concentrated to an appropriate cell mass, dried by freeze-dryer, and then stored into a deep freezer until use. The prepared ghost cells were administered into mice by appropriate dilution using BSG buffer (PBS buffer containing 0.1% gelatin).

**Mouse vaccination by ghost cell**

Freeze-dried ghost cells were serially diluted by ten-folds, and were administered via oral route into BALB/c mice acclimatized during 1 week. The inoculated dosages were adjusted to 1×10^7 CFU/ml. Food and water were prohibited

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**Table 1. Bacterial strains and plasmids used in this study**

| Strains and plasmids | Descriptions | References |
|----------------------|--------------|------------|
| **E.coli**           |              |            |
| Top10                | F-mcrA (mrr-hsdRMS-mcrBQ) ψ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK rpsL (Str')endA1 fhuA2 Δ(argF-lacZ)U169 phoA glnV44 ΔlacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17 | Invitrogen |
| DH5α                 |              | Invitrogen |
| χ6212                | ϕ 80d lacZ ΔM15 deor Δ(lacZYA-argF)U169 supE44 Δ gyrA96 recA1 relA1 endA1 asdA4 Δ zhf-2::Tn10 hsdR17 (R'M') | [12, 13] |
| **Salmonella**       |              |            |
| S.Typhimurium χ3339 | wild-type strain SL1344, hisG rpsL | [12, 13] |
| S.Typhimurium χ8554 | X3339 asdA16 | [12, 13] |
| S.Typhimurium JOL389| wild-type strain isolated from swine in South Korea | Chonbuk National University |
| **Plasmids**         |              |            |
| pMMP99               | a derivative of T-vector carrying cI857 PpE | Gyeongnam National University of Science and Technology |
| pMMP184              | a derivative of pYA3342 carrying cI857 PR E ara and Pp ompA TM | Gyeongnam National University of Science and Technology |
| pMMP300              | a derivative of pMMP184 carrying LT-B | Gyeongnam National University of Science and Technology |
for four hours before administration of the ghost cells, and supplied at one hour after administration ad libitum. The vaccinated mice were observed for 10 weeks post administration. The peripheral blood and vaginal lavage or extract of feces to measure IgG and IgA amounts, respectively, were collected for 0 - 10 weeks from vaccinated mice by the ghost cells. The cytokines were measured from spleen harvested at 35 days post administration.

**ELISA (Enzyme-linked immunosorbent assay)**

ELISA was performed according to method of Ha et al. [6, 7]. LPS (0.2 mg/ml) was dissolved in 0.05 M carbonate buffer (pH 9.6), and then coated on microtiter plate for overnight at 4°C. The coating solution was removed from the treated microtiter plate (SPL) and washed 4~6 times with PBS buffer (pH 7.4). The treated plate was blocked for 1 hr at 37°C by PBS buffer including 0.1% skim milk. The wells were reacted for 2 hr at 37°C with plasma, fecal extract, and vaginal lavage solution diluted by serially ten folds. The reactions were followed with goat anti-mouse IgG-, IgG1-, IgG2a-, and IgA-horseradish peroxidase (HRP)-conjugate (Southern Biotech) at 1:5,000 for 2 hr. The bound HRP was reacted for 10~90 min using 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS, Sigma-Aldrich). Antibody titers were determined by 405 nm in wavelength with an ELISA reader (Dynex, USA) after the reaction was stopped with 0.1% SDS.

**Protection of mice immunized with ST ghost cells against virulent ST**

In order to examine protection against virulent ST to mice which immunized by the ST ghost cells, challenge test was performed as follows. The ghost cells carrying each antigen were administrated by oral route with 1×10⁷ CFU/ml dose, and boosting was done by same dose after 2 weeks. The challenge test was performed by oral route with 1.2×10⁷ CFU/ml dosage of ST X3339 at 2 weeks post boosting ghost administration. Food and water was prohibited for four hours before administration of bacteria and supplied at one hour after administration ad libitum. The treated mice were observed for 4 weeks. The animal experiments adapted in this study were conducted under approval from the Gyeongnam National University of Science and Technology Animal Ethics Committee in accordance with the guidelines of the Korean Council on Animal Care (AEC-20100730-0002).

**Statistics analysis**

Individual comparisons among least squares means (LSM) for significant differences were made according to the multiple range test of Duncan. All analyses were performed within the SAS statistical software package (version 9.1, SAS Inst., Inc., USA), and differences were considered significant at $p<0.05$.

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![Fig. 1. Total IgG immune responses according to ghost strains via oral route of BALB/c. LPS and IgG conjugated with HRP was treated for 2 hr at room temperature by 2 ug/ml concentration and by 1:2,000 dilution, respectively. The treated solutions were measured at 405 nm by ELISA reader. X- and Y-axes indicate week post injection and immune response by log value, respectively. Con; oral administration by only PBS, A; ST X8554 [pMMP184], B; ST X8554 [pMMP184] +JOL389. Values are significantly different ($p<0.05$) between the same sample treated groups. Values are significantly different ($p<0.05$) in the same week from each other.](image-url)
Results and Discussion

Immune response of total IgG for a combined ST ghost vaccine
To construct a bacterial ghost vaccine, it is often applied by a system that combines bacteriophage lambda cII857 PR and ΦX174 E gene [21]. In this study, we induced the formation of ST ghost cells by using the cII857 PR::E system as previous study [6, 7, 21]. Otherwise, we constructed and applied pMMP184 through introduction of aspartate semialdehyde dehydrogenase (asl) gene used in the host-balanced lethal system [6, 7], instead of the antibiotic marker that was used for the selection of strains in previous studies [10, 14, 17].

Generally, since LT-B is evaluated to have an excellent effect as an adjuvant, it has been used as an adjuvant for immune enhancement of target animals [3, 11, 18]. On the other hand, Bacterial ghost not only induces function of the

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Fig. 2. IgG1 immune responses according to ghost strains via oral route of BALB/c. LPS and IgG1 conjugated with HRP was treated for 2 hr at room temperature by 2 μg/ml concentration and by 1:2,000 dilution, respectively. The treated solutions were measured at 405 nm by ELISA reader. X- and Y-axes indicate week post injection and immune response by log value, respectively. Con; oral administration by only PBS, A; ST x8554 [pMMP184], B; ST x8554 [pMMP184] +JOL389. **Values are significantly different (p<0.05) in the same week from each other.

Fig. 3. IgG2a immune responses according to ghost strains via oral route of BALB/c. LPS and IgG2a conjugated with HRP was treated for 2 hr at room temperature by 2 μg/ml concentration and by 1:2,000 dilution, respectively. The treated solutions were measured at 405 nm by ELISA reader. X- and Y-axes indicate week post injection and immune response by log value, respectively. Con; oral administration by only PBS, A; ST x8554 [pMMP184], B; ST x8554 [pMMP184] +JOL389. **Values are significantly different (p<0.05) in the same week from each other.
immune response, but also play a role as an adjuvant [19]. From previously studies of our laboratory, we revealed abilities of immune responses similar to LT-B in the case of administration of the combined ghost cells via intramuscular route, which the combined groups contain ST MMP13 [pMMP184], MMP13 [pMMP184]/MMP13 [pMMP300], X8554 [pMMP184], X8554 [pMMP184]/X8554 [pMMP300], X8554 [pMMP184]/X3339, and X8554 [pMMP184]/JOL389 [6, 7]. In the present study, a group of vaccines selected from intramuscular route of previous study was orally administered with and without ST JOL389 for analyzing the immune responses. As a result, the immune response to the total IgG of ST X8554 [pMMP184]/JOL389 group from 2 weeks post-vaccination was detected as shown in Fig. 1. The immune response in single ST X8554 [pMMP184] group was delayed up to 6 weeks post third administration. However, the combined group was elicited at 2 weeks post primary administration. At 10 weeks, as compared with the case of using X8554 [pMMP184], IgG immune response of ST X8554 [pMMP184]/JOL389 was showed by value of 8 folds more than that of ST X8554 [pMMP184].

Fig. 4. Vaginal IgA immune responses according to ghost strains via oral route of BALB/c. LPS and IgA conjugated with HRP was treated for 2 hr at room temperature by 2 ug/ml concentration and by 1:2,000 dilution, respectively. The treated solutions were measured at 405 nm by ELISA reader. X- and Y-axes indicate week post injection and immune response by log value, respectively. Con; oral administration by only PBS, A; ST X8554 [pMMP184], B; ST X8554 [pMMP184] +JOL389. **X** Values are significantly different (p<0.05) between the same sample treated groups.

Fig. 5. Fecal IgA immune responses according to ghost strains via oral route of BALB/c. LPS and IgA conjugated with HRP was treated for 2 hr at room temperature by 2 ug/ml concentration and by 1:2,000 dilution, respectively. The treated solutions were measured at 405 nm by ELISA reader. X- and Y-axes indicate week post injection and immune response by log value, respectively. Con; oral administration by only PBS, A; ST X8554 [pMMP184], B; ST X8554 [pMMP184] +JOL389.
Immune responses of IgG subtypes and secretory IgA for the combined ghost vaccine

Productions of IgG1 and IgG2a subtypes of immunoglobulin IgG are promoted by Th2 and Th1, respectively, which induce humoral and cell-mediated immune responses [4,5,16]. We analyzed the content of IgG1 and IgG2a to examine the humoral and cell-mediated immune responses. As shown in Fig. 2, IgG1 was difficult of observing the specific difference between the two groups, but both groups were elevated the amount of IgG1 from 4 weeks post-vaccination. The IgG2a was observed in the differential amount after 4, 6, and 10 weeks (Fig. 3). In comparison to the control group, vaginal sIgA was raised at 4-8 weeks post-vaccination (Fig. 4). ST χ8554 [pMMP184]/JOL389 group was detected generally high amount of vaginal sIgA in comparison with the ST χ8554 [pMMP184] group. Although fecal sIgA was shown a little difference in 8 weeks, it is estimated that the detection of sIgA was not significant difference (Fig. 5).

A protective effect in mice vaccinated with ST ghost cells against virulent ST

To assess the protection for the mice immunized with the ghost cells against virulent ST, wild-type ST χ3339 (1.2×10⁷ CFU), which is higher dose of 10-fold than that of LD50 [12, 13], was administrated orally at 4 weeks post-immunization. The results of challenge assay using ST χ3339 showed mortality of 50% in the control group, but ST χ8554 [pMMP184] and χ8554 [pMMP184]/JOL389 groups exhibited a protective effect of 100% (Fig. 6).

In summary, when the combined vaccine composed of ST χ8554 [pMMP184]/S. Typhimurium JOL389 was orally administered in mice, the ability of immune induction was superior to a single group of ST χ8554 [pMMP184]. Furthermore, the protection of mice against virulent S. Typhimurium χ3339 increased above 50% when compared with control group. Therefore, we conclude that the vaccine is a possible of application as a ST ghost vaccine.

Acknowledgement

This research was supported by Gyeongnam National University of Science and Technology Grant 2014.

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초록: 복합 살모넬라 타이피무리움 고스트 백신의 마우스 구강 투여에 의한 면역 응답

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살모넬라 타이피무리움 JOL389와 χ3339는 마우스에 강한 독력을 가진 균주들이며, X8554는 X3339로부터 유도된 플라스미드 pMMP184가 제조된 후에, BALB/c 마우스의 구강 경로를 경유하여 투여되었다. 총 IgG의 함량 변화는 χ8554 고스트 세포의 부스팅으로 발현 함량이 낮게 나타났지만, 3차 접종의 2주 경과 후, 6주차에서 증가되는 양상을 보였다. 그러나, 혼합 백신 그룹인 JOL389/χ8554 그룹에서는 총 IgG의 함량이 일차 접종 후 2주차부터 상승되는 경향을 보였고, 추가접종이 진행되면서 많은 상승 폭을 나타내었다. 총 IgG의 함량은 백신 접종 후 10주차에서 X8554 그룹에 비교하여 JOL389/χ8554는 8배 이상 높은 것으로 관찰되었다. IgG1, IgG2a, 분비 IgA의 함량은 백신화 후 4주차에서 상승되었다. 독력 살모넬라 타이피무리움 X3339로도 전실험결과, X8554 [pMMP184]과 X8554 [pMMP184]/JOL389는 대조군에 비교하여 50% 이상의 보호효과가 관찰되었다. 이들 결과는 X8554 [pMMP184]/JOL389는 X8554 [pMMP184]보다 더 높은 면역 응답을 유도하는 것이 가능한 것으로 추정된다.