NOTE *Bacteriology*

Immunological study of an attenuated *Salmonella* Typhimurium expressing ApxIA, ApxIIA, ApxIIIA and OmpA of *Actinobacillus pleuropneumoniae* in a mouse model

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ABSTRACT. *Salmonella* Typhimurium strain expressing the *Actinobacillus pleuropneumoniae* antigens, ApxIA, ApxIIA, ApxIIIA and OmpA, was previously constructed as a vaccine candidate for porcine pleuropneumonia. This strain was a live attenuated (Δ*lon*Δ*cpxR*Δ*asd*)*Salmonella* as a delivery host and contained a vector containing *asd*. An immunological study of lymphocyte proliferation, T-lymphocyte subsets and cytokines in the splenocytes of a mouse model was carried out after stimulation with the candidate *Salmonella* Typhimurium by intranasal inoculation. The splenic lymphocyte proliferation and the levels of IL-4, IL-6 and IL-12 of the inoculated mice were significantly increased, and the T- and B-cell populations were also elevated. Collectively, the candidate may efficiently induce the Th1- and Th2-type immune responses.

KEY WORDS: *Actinobacillus pleuropneumoniae*, porcine pleuropneumonia, *Salmonella* Typhimurium mutant

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Porcine pleuropneumonia (PP) is a severe and contagious swine pulmonary disease caused by *Actinobacillus pleuropneumoniae* (APP) [2]. This disease affects pigs of all ages and has a major impact on economics, ecology and animal welfare in the pig-rearing industry [2, 7]. For control of PP, vaccination is important [7]. APP is divided into 15 serotypes [1] with a lack of cross-protection between the main serotypes, [1] thus resulting in slow progress in the development of vaccines [19]. Among several APP virulence factors, Apx exotoxins are recognized as major contributors to virulence [7]. The importance of Apx toxins in protective immunity against PP has been demonstrated in many studies [7, 17]. APP produces three virulent Apx toxins, ApxI, ApxII and ApxIII [1]. An outer membrane protein, OmpA, is a highly conserved protein that is an integral component of the outer membranes of Gram-negative bacteria. It has characteristic heat-modifiable properties and is also immunogenic [14]. In a previous study [10], new live attenuated *Salmonella* Typhimurium strains expressing each ApxIA, ApxIIA, ApxIIIA and OmpA antigen as vaccine candidates for the prevention of PP were constructed. In addition, we confirmed that the intranasal immunization with the vaccine candidate effectively stimulated both antigen-specific IgG and IgA immune responses in the systemic and mucosal compartments, respectively [10]. The vaccine candidate effectively protected APP infection after challenge with a wild-type APP in mice [10]. In order to understand the further immunological responses after intranasal immunization with this candidate, the proliferation of lymphocytes, T-lymphocyte subsets and the resulting cytokines was studied in a murine model.

The recombinant ApxIA, ApxIIA, ApxIIIA and OmpA proteins were prepared from JOL997, JOL1000, JOL1007 and JOL1076 strains, respectively, using an affinity purification process with nickel-nitrilotriacetic acid-agarose (Qiagen, Valencia, CA, U.S.A.) [10]. The delivery strains, attenuated *Salmonella* Typhimurium (Δ*lon*Δ*cpxR*Δ*asd*) mutant JOL1300 for ApxIA, JOL1301 for ApxIIA, JOL1302 for ApxIIIA and JOL1303 for OmpA, were grown individually in LB broth at 37°C to an OD_{600} of 0.8 [10]. The cells were harvested by centrifugation and were resuspended to 1×10^7 colony-forming units (CFU)/m*l* in sterile phosphate buffered saline (PBS). Thirty female BALB/c mice were divided equally into two groups. All mice were intranasally inoculated at 6 weeks of age [0 week post inoculation (WPI)]. Group A mice were inoculated with approximately 1×10^5 CFU in 10 μl of an equal-volume mixture of the four delivery strains (consisting of approximately 0.25×10^5 CFU per strain in 10 μl). Group B mice were inoculated with 10 μl of sterile PBS, as previously described [10]. The animal experiments mentioned in this study were conducted with ethics approval (CBU 2011-0017) from the Chonbuk National University Animal Ethics Committee in accordance with the guidelines of the Korean Council on Animal Care.

Spleens from 5 mice per group were aseptically collected at 4 WPI as previously described [5]. The splenocyte proliferation assay was performed with specific antigens as previously described [18], with a slight modification. Briefly, 100 μl of cell suspension at 5×10^6 cells/m*l* in complete RPMI-1640 medium were incubated in 96-well tissue culture plates with 50 μl of, medium alone as an unstimulated control or medium containing 4 μg/m*l* of ApxIA, ApxIIA, ApxIIIA or OmpA antigen at 37°C in a humidified 5% CO_2 atmosphere for 48 hr. Concavalin A (0.5μg/well) was used

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as a positive control. The blastogenic response for the assay was expressed as the mean stimulation index (SI), calculated by dividing the value of stimulated culture with the antigens by the value of non-stimulated culture. The percentage (%) difference in stimulated and unstimulated splenocytes of each antigen was compared. To analyze changes in the T-lymphocyte subsets in the spleen after inoculation, the splenocytes from five mice in each group were collected at 4 WPI following the method described above. Subsequently, the cells were stimulated with the individual antigen for 12 hr [13]. The cells were incubated with monoclonal antibodies against CD3, CD4, CD8 and CD45R (eBioscience, San Diego, CA, U.S.A.), as previously described [5], and analysis was carried out using a FACS CaliburTM (Becton Dickinson Immunocytometry Systems, San Jose, CA, U.S.A.) instrument equipped with a 15 mW, 488 nm air-cooled argon laser. Data analysis was performed using FlowJo software (Treestar, Inc., San Carlos, CA, U.S.A.). Approximately 5 $\times 10^6$ splenocyte cells per well were cultured in 24-well tissue culture plates for 72 hr in the presence of 4 μg/m*l* of each antigen [5]. The cell suspensions were centrifuged at 1,500 rpm for 5 min to obtain the supernatants. ELISA was also used to measure the concentration of cytokines, such as IL-4, IL-6 and IL-12 in the supernatants using the mouse cytokine ELISA Ready-SET-GO reagent set according to the manufacturer's instructions (eBioscience Inc.). All data were expressed as the mean ± standard deviation (SD). Analyses were performed with SPSS version 16.0 software (SPSS, Chicago, IL, U.S.A.). An independent sample *t* test was used to analyze statistical differences in the immune responses between the immunized groups and an unimmunized control group. Statistical significance was determined at $P \leq 0.05$.

Splenocyte proliferation responses against individual antigens revealed a significant increase in the SI values of the inoculated group mice compared to those of the control group mice ($P \leq 0.05$). In group A, SI values against the ApxIA, ApxIIA, ApxIIIA and OmpA antigens were 1.36 ± 0.17, 1.42 ± 0.19, 1.48 ± 0.10 and 1.45 ± 0.10, respectively, while SI values in group B were 1.01 ± 0.03, 1.02 ± 0.05, 1.08 ± 0.11 and 1.11 ± 0.13, respectively (Table 1). Analysis of the T-cell populations against ApxIA, ApxIIA, ApxIIIA and OmpA in group A showed an increased number of $CD3^+CD4^+$ and $CD3^+CD8^+$ lymphocytes compared to those of the control group. In addition, the B-lymphocytes ($CD45R^+$) against the proteins in group A were increased (Fig. 1). The levels of IL-6 and IL-12 ($P<0.01$), as well as IL-4 ($P<0.05$) in the inoculated group were significantly increased compared to those, in the non-inoculated group (Fig. 2).

For complete pathogen clearance, the adaptive immune response is stimulated, in which specific antibodies, such as IgA and IgG, play a major role in preventing extracellular bacteria, such as APP [15]. Activated $CD4^+$ T-helper lymphocytes provide stimulation for specific antibodies, which function to neutralize bacterial toxins, such as ApxIA, ApxIIA and ApxIIIA, block attachment of APP to host cells and mediate phagocytosis of invading the microorganisms [15]. Attenuated *Salmonella* Typhimurium is being widely studied as a delivery system for targeted antigens, and this vehicle has been shown to effectively induce mucosal and systemic immune responses to heterologous antigens in animals [9]. In our previous study, intranasal immunization with the vaccine candidate effectively induced both antigen-specific IgG and IgA immune responses [10]. In addition, the antigen-specific cell-mediated immune response is an important factor in the preventing and clearing bacterial infection [15]. The antigen-specific cell-mediated immune responses can be numerically expressed as a measurement of antigen-specific lymphocyte proliferation activity [16]. The antigen-specific lymphocyte proliferation assay indicated that significant cell-mediated immune responses were elevated in the inoculated group with the delivery strain-based vaccine candidate. The antigen-specific lymphocyte populations in the splenocytes were examined to gain more insight into the immunological effects of intranasal inoculation with the candidate. In particular, the efficient proliferation of $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells in splenocytes suggests that intranasal inoculation promotes T cell-mediated immune responses. Additionally, antigen-specific $CD45R^+$ cells (B cells) in the splenocytes were increased after inoculation.

Table 1. Splenocyte proliferative responses against ApxIA, ApxIIA, ApxIIIA and OmpA antigens in inoculated group A and control group B mice

Antigen	Group	SPA (SI value)
ApxIA	A	1.36 ± 0.17 a)
	B	1.01 ± 0.03
ApxIIA	A	1.42 ± 0.19 a)
	B	1.02 ± 0.05
ApxIIIA	A	1.48 ± 0.10 a)
	B	1.08 ± 0.11
OmpA	A	1.45 ± 0.10 a)
	B	1.11 ± 0.13

SPA, Splenocyte proliferative assay. SI, the stimulation index. Asterisks indicate significant differences between the values of the immunized and control groups. a) $P \leq 0.05$.

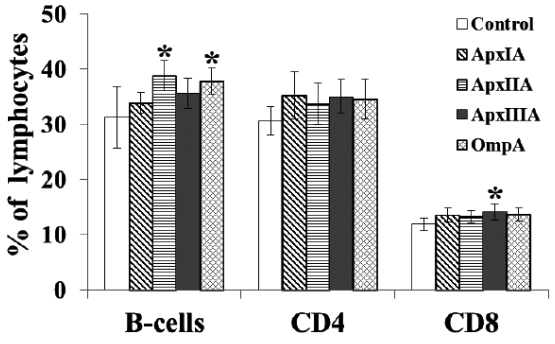

Fig. 1. Flow cytometric analysis of T-lymphocyte and B-lymphocyte subpopulations in the spleen at 4 WPI. Bar graphs represent B-cell, $CD3^+CD4^+$ and $CD3^+CD8^+$ splenocyte subpopulations in the inoculated mice. All values of the bar graphs are presented as the mean ± SD. Asterisks indicate significant differences between the values of the inoculated and control groups (*$P<0.05$).

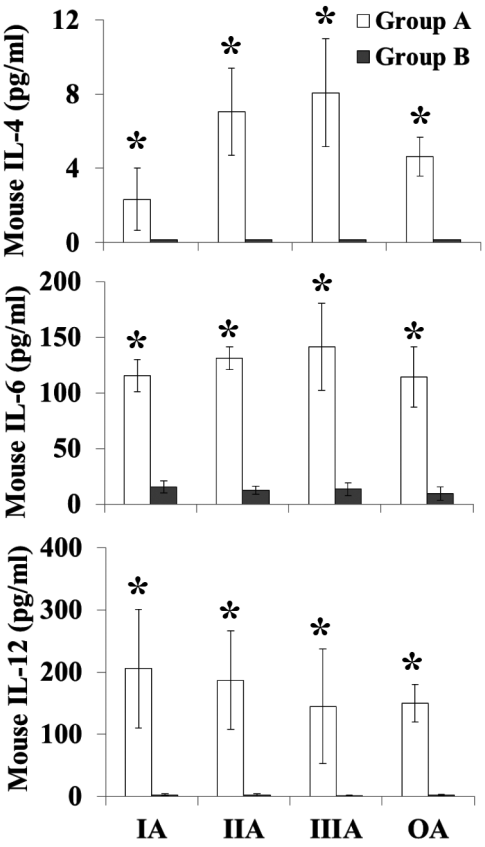

Fig. 2. Cytokine titers in the splenocytes at 4 WPI. Group A, (□); group B, (■). Data are the mean of all mice in each group; error bars show SD. Asterisks indicate a significant difference between the values of group A mice (*P<0.05) and those of the control group mice.

These data imply that intranasal immunization with the candidate may induce immunological reactions associated with T cell-dependent B cell activation in response to APP.

IL-4 activates B cells and induces a T-helper 2 (Th2) type immune response, while IFN-γ induces a Th1 type immune response [6]. IL-12 is an immune regulatory cytokine [12]. Furthermore, IL-12 has been shown to play a crucial role in the development of Th1 type immunity [3, 12]. A Th1 antibody response is associated with protection from infection, while a Th2 response is helpful for clearing infections [6]. Thus, an ideal vaccine should enhance Th1 responses while concomitantly maintaining Th2 responses. In this study, the concentrations of IL-4 and IL-12 in the culture media of *in vitro* stimulated splenocytes from the immunized group mice were significantly increased. These results suggest that intranasal immunization with the candidate can induce both Th1- and Th2-type immune responses.

Secretory IgA transported and secreted across the mucosal epithelium into the lumen can inhibit the attachment of microorganisms and/or neutralize exotoxins [8]. The APP bacteria are located in the tonsils of infected animals during the infectious stage, which can allow the bacteria to escape from host immune surveillance [4]. The defense mechanism against pulmonary bacterial infections depends on the clearance of the pathogens from the respiratory tract, and mucosal immunity from IgA production is necessary for effective vaccination against PP [7]. In previous reports, however, it has been shown that systemic immunization via intramuscular or subcutaneous injection does not significantly induce a mucosal IgA response [7, 17]. Recently, it has been assessed that the induction of the mucosal immune response through intranasal immunization with live attenuated vaccines mediates secretory IgA production and can prevent both disease outbreak as well as the generation of asymptomatic carriers by precluding pathogen attachment to the tonsils [7]. Generally, T-helper 2 (Th2) cells produce IL-4, IL-5, IL-6 and IL-10 cytokines [11]. Although IL-4 is important for mucosal IgA induction, IL-5, IL-6 and IL-10 can act as a counterbalance to IL-4 in the presence of a strong Th1 response [20]. In our previous study, we confirmed that the intranasal inoculation procedure effectively stimulated IgA immune responses in mucosal compartments [10], and the results in this study showed that the levels of IL-6 against these antigens in the splenocytes of the inoculated group were significantly increased. This result implies that intranasal inoculation with the candidate may strongly induce mucosal IgA via IL-6 in the presence of a strong Th1 response. In addition, we investigated cytokines to each antigen from mice inoculated with delivery *Salmonella* strain containing vector only without each antigen gene. The results showed that IL-4, IL-12 and IL-6 cytokine concentrations to the individual antigens were not effectively induced (data not shown) by delivery *Salmonella* strain containing vector only unlikely the vaccine candidate. These results indicate that the cytokines against the individual antigens can be effectively induced by each antigen expressed from the delivery *Salmonella* strains, but not *Salmonella* itself.

Collectively, these results help to understand that intranasal immunization with the vaccine candidate effectively protected APP infection through induction of cell-mediated immune response as well as serum IgG and mucosal IgA immune responses.

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REFERENCES

1. Blackall, P. J., Klaasen, H. L., van den Bosch, H., Kuhnert, P. and Frey, J. 2002. Proposal of a new serovar of *Actinobacillus pleuropneumoniae*: serovar 15. *Vet. Microbiol.* **84**: 47–52. [Medline] [CrossRef]
2. Bossé, J. T., Janson, H., Sheehan, B. J., Beddek, A. J., Rycroft, A. N., Kroll, J. S. and Langford, P. R. 2002. *Actinobacillus pleuropneumoniae*: pathobiology and pathogenesis of infection. *Microbes Infect.* **4**: 225–235. [Medline] [CrossRef]
3. Brumme, S., Arnold, T., Sigmarsson, H., Lehmann, J., Scholz,

H. C., Hardt, W. D., Hensel, A., Truyen, U. and Roesler, U. 2007. Impact of *Salmonella* Typhimurium DT104 virulence factors invC and sseD on the onset, clinical course, colonization patterns and immune response of porcine salmonellosis. *Vet. Microbiol.* **124**: 274–285. [Medline] [CrossRef]

4. Chiers, K., De Waele, T., Pasmans, F., Ducatelle, R. and Haesebrouck, F. 2010. Virulence factors of *Actinobacillus pleuropneumoniae* involved in colonization, persistence and induction of lesions in its porcine host. *Vet. Res.* **41**: 65. [Medline] [CrossRef]
5. Chin'ombe, N., Bourn, W. R., Williamson, A. L. and Shephard, E. G. 2009. An oral recombinant *Salmonella enterica* serovar Typhimurium mutant elicits systemic antigen-specific CD8+ T cell cytokine responses in mice. *Gut Pathog.* **1**: 9. [Medline] [CrossRef]
6. Delves, P. J. and Roitt, I. M. 2000. The immune system. Second of two parts. *N. Engl. J. Med.* **343**: 108–117. [Medline] [CrossRef]
7. Haesebrouck, F., Pasmans, F., Chiers, K., Maes, D., Ducatelle, R. and Decostere, A. 2004. Efficacy of vaccines against bacterial diseases in swine: what can we expect? *Vet. Microbiol.* **100**: 255–268. [Medline] [CrossRef]
8. Holmgren, J. and Czerkinsky, C. 2005. Mucosal immunity and vaccines. *Nat. Med.* **11** Suppl: S45–S53. [Medline] [CrossRef]
9. Hur, J. and Lee, J. H. 2011. Immune responses to new vaccine candidates constructed by a live attenuated *Salmonella* Typhimurium delivery system expressing *Escherichia coli* F4, F5, F6, F41 and intimin adhesin antigens in a murine model. *J. Vet. Med. Sci.* **73**: 1265–1273. [Medline] [CrossRef]
10. Hur, J. and Lee, J. H. 2014. Optimization of immune strategy for a construct of *Salmonella*-delivered ApxIA, ApxIIA, ApxIIIA and OmpA antigens of *Actinobacillus pleuropneumoniae* for prevention of porcine pleuropneumonia using a murine model. *Vet. Res. Commun.* **38**: 87–91. [Medline] [CrossRef]
11. Kramer, D. R., Sutherland, R. M., Bao, S. and Husband, A. J. 1995. Cytokine mediated effects in mucosal immunity. *Immunol. Cell Biol.* **73**: 389–396. [Medline] [CrossRef]
12. Lehmann, J., Bellmann, S., Werner, C., Schröder, R., Schütze, N. and Alber, G. 2001. IL-12p40-dependent agonistic effects on the development of protective innate and adaptive immunity against *Salmonella* enteritidis. *J. Immunol.* **167**: 5304–5315. [Medline] [CrossRef]
13. Li, Y., Wang, S., Xin, W., Scarpellini, G., Shi, Z., Gunn, B., Roland, K. L. and Curtiss, R. 3rd. 2008. A sopB deletion mutation enhances the immunogenicity and protective efficacy of a heterologous antigen delivered by live attenuated *Salmonella enterica* vaccines. *Infect. Immun.* **76**: 5238–5246. [Medline] [CrossRef]
14. Mahasreshti, P. J., Murphy, G. L., Wyckoff, J. H. 3rd., Farmer, S., Hancock, R. E. and Confer, A. W. 1997. Purification and partial characterization of the OmpA family of proteins of *Pasteurella haemolytica*. *Infect. Immun.* **65**: 211–218. [Medline]
15. Moyle, P. M., McGeary, R. P., Blanchfield, J. T. and Toth, I. 2004. Mucosal immunisation: adjuvants and delivery systems. *Curr. Drug Deliv.* **1**: 385–396. [Medline] [CrossRef]
16. Rana, N. and Kulshreshtha, R. C. 2006. Cell-mediated and humoral immune responses to a virulent plasmid-cured mutant strain of *Salmonella enterica* serotype gallinarum in broiler chickens. *Vet. Microbiol.* **115**: 156–162. [Medline] [CrossRef]
17. Seo, K. W., Kim, S. H., Park, J., Son, Y., Yoo, H. S., Lee, K. Y. and Jang, Y. S. 2013. Nasal immunization with major epitope-containing ApxIIA toxin fragment induces protective immunity against challenge infection with *Actinobacillus pleuropneumoniae* in a murine model. *Vet. Immunol. Immunopathol.* **151**: 102–112. [Medline] [CrossRef]
18. Yamamoto, S., Wada, J., Katayama, T., Jikimoto, T., Nakamura, M., Kinoshita, S., Lee, K. M., Kawabata, M. and Shirakawa, T. 2010. Genetically modified Bifidobacterium displaying *Salmonella*-antigen protects mice from lethal challenge of *Salmonella* Typhimurium in a murine typhoid fever model. *Vaccine* **28**: 6684–6691. [Medline] [CrossRef]
19. Yang, F., Ma, Q., Lei, L., Huang, J., Ji, Q., Zhai, R., Wang, L., Wang, Y., Li, L., Sun, C., Feng, X. and Han, W. 2014. Specific humoral immune response induced by *propionibacterium acnes* can prevent *Actinobacillus pleuropneumoniae* infection in mice. *Clin. Vaccine Immunol.* **21**: 407–416. [Medline] [CrossRef]
20. Yang, Y., Zhang, Z., Yang, J., Chen, X., Cui, S. and Zhu, X. 2010. Oral vaccination with Ts87 DNA vaccine delivered by attenuated *Salmonella* typhimurium elicits a protective immune response against *Trichinella spiralis* larval challenge. *Vaccine* **28**: 2735–2742. [Medline] [CrossRef]