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# *Atractylodis macrocephalae Koidz.* polysaccharides enhance both serum IgG response and gut mucosal immunity

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ABSTRACT

Fifty-six mice were randomly divided into four groups with 14 mice in each. Two groups were subcutaneously injected twice with a foot-and-mouth disease vaccine with 2-week intervals; each of them had been orally administered 0.89% saline or *Atractylodis macrocephalae Koidz.* polysaccharides (RAMPS) 0.05 g for 4 days before immunization. The rest were not immunized but treated in the same way. One-week after the primary and two weeks after the booster immunization, half in each group were sacrificed to measure serum IgG and the parameters for the intestinal mucosal immunity. Results indicated that oral administration of RAMPS increased both serum specific IgG response and intestinal mucosal immunity as shown by elevated total sIgA, mRNA expression of TGF-β, IL-6, TNF-α, IgA$^+$ cells and intestinal intraepithelial lymphocytes in duodenum. It is suggested that increased serum IgG response may be associated with enhanced local mucosal immunity by oral administration of RAMPS.



## 1. Introduction

*Atractylodis macrocephalae Koidz.* is a plant in Compositae family with plenty of natural resources in China. It is traditionally used as a digestive stimulator in Chinese medicine. The rhizome of this plant (RAM) contains three categories including volatile oil, lactones and polysaccharides (Duan, Xu, Liu, & Li, 2008). RAM has been reported to have immunomodulatory, antitumor activities and anti-inflammatory properties (Li, He, Dong, & Jin, 2007; Mao, Lu, Zeng, & Ruan, 1996; Zhang, Zhang, Shi, & Liu, 2006). Our previous studies have demonstrated that oral administration of a decoction made from RAM has significantly increased the immune response induced by a foot-and-mouth disease vaccine in mice (Li, Sakwiwatkul, Yutao, & Hu, 2009). Further investigation has found that the oral adjuvant activities of RAM may be attributed to the polysaccharides (RAMPS) of the plant (Xie, Li, Su, & Hu, 2012). Due to its high molecular weight, RAMPS is supposed to show its immunomodulatory activity not being directly absorbed into the blood circulation but by triggering the enteric mucosal immunity.

The enteric mucosal system is a physical barrier to prevent microbial infections and plays an important role in maintaining homeostasis (Kagnoff, 1996; Yuan & Walker, 2004). The system consists of many immunocompetent cells and molecules scattered throughout lamina propria and organized lymphatic tissues (Zhao et al., 2010). Oral route is a traditional way for administration of Chinese medicinal herbs. The immunomodulatory activities of the herbs might associate with the mucosal immune system. However, it remains unclear whether RAMPS has any effect on the enteric mucosal immune response. The aim of this study was to investigate the mouse enteric mucosal immunity after oral administration of RAMPS by measuring intestinal total IgA, mRNA expression, IgA$^+$ cells and intraepithelial lymphocytes (IELs) in the intestinal tissues.

## 2. Materials and methods

### 2.1. Animals

Female ICR mice (5 weeks old) weighing 18–22 g were purchased from Shanghai Laboratory Animal Center (SLAC) Co. Ltd. (Shanghai, China), and housed in polypropylene cages with sawdust bedding in hygienically controlled environment with a temperature of 24 ± 1 °C, humidity of 50 ± 10%, and a 12/12 h light/dark cycle. Feed and water were supplied ad libitum. All procedures related to the animals and their care conformed to the internationally accepted principles as found in the Guidelines for Keeping Experimental Animals issued by the government of China.

### 2.2. Chemicals and reagents

Goat anti-mouse IgG horseradish peroxidase conjugate was from Kierkegaard & Perry Laboratories, Inc., USA;

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3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co., USA; rabbit anti-mouse IgA was from Novus Biological Co., USA; goat anti-rabbit IgG horseradish peroxidase conjugate was from Santa Cruz Biotechnology Inc., USA; ROX Reference Dye was from Invitrogen Co., USA; EvaGreen™ Dye was from Biotium Inc., USA; RNAiso™ plus was from TaKaRa Biotechnology (Dalian) Co., Ltd. China; revert Aid™ M-MuLV reverse transcriptase was from Fermentas, USA; diethylpyrocarbonate (DEPC) and ribonuclease inhibitor was from Biobasic, Canada; Oligo (dT)18 was from Sangon, China; mouse IgA ELISA kit was from eBioscience Inc., USA.

### 2.3. Atractylodis macrocephalae Koidz. polysaccharides (RAMPS)

Dried rhizome of *Atractylodis macrocephalae Koidz.* (RAM) was purchased from Hu Qing Yu Tang Co. Ltd, Hangzhou, China. RAMPS was extracted and purified as previously described (Xie et al., 2012), and it was composed of rhamnose (4.92 mol.%), arabinose (12.25 mol.%), xylose (10.17 mol.%), mannose (24.32 mol.%), glucose (55.74 mol.%), and galactose (6.65 mol.%) with the molar ratio of 1.00:2.49:2.07:4.94:11.33:1.35, respectively.

### 2.4. Immunization and oral administration of RAMPS

Fifty-six female ICR mice were randomly divided into four groups with 14 mice in each. Groups I and III were orally administered for 4 days with 0.25 ml of RAMPS (0.05 g) solution; groups II and IV were administered with 0.89% saline solution in the same way. Groups I and II were subcutaneously (S.C.) injected twice with 200 μl of FMDV type O vaccine (Lanzhou Veterinary Research Institute, China). The treatment was performed twice with 2-week intervals.

### 2.5. Sample collection

One week after the first immunization and two weeks after the booster immunization, half of mice in each group were under halothane anesthesia to collect blood samples by orbital venous puncture for detection of specific IgG levels. And after killing by cervical vertebra dislocation, their guts and fecal samples were immediately collected following a previously reported method (Florindo et al., 2009; Li, Takeda, & Miyamura, 2001). Briefly, a gut fraction was removed; longitudinally sectioned, fecal samples were scraped and extracted by making a 1:10 suspension (w/v) in PBS containing 0.1 mg/ml trypsin inhibitor, 50 mM EDTA, 0.1% BSA, and 40 μM PMSF. All samples were vortexed to disrupt all solid material, centrifuged at 12,000 × *g* for 20 min at 4 °C, and the supernatants were analyzed for total intestinal IgA levels; part of the duodenum (about 200 μg) was grinded with liquid nitrogen, lysed in RNAiso™ Plus reagent and the total RNA was isolated for determination of cytokine mRNA expression; tissue samples were fixed in 10% neutral buffered formalin (NBF) for 24 h for routine histology.

### 2.6. Serum FMDV-specific IgG and intestinal total IgA

An indirect enzyme-linked immunosorbent assay (ELISA) was conducted to measure serum FMDV-specific IgG as previously described (Song, Bao, Wu, & Hu, 2009). Total intestinal IgA was quantified according to the manufacturer's protocol. Briefly, the 96-well corning costar plates were coated with 100 μl/well of purified anti-mouse IgA monoclonal antibody diluted in phosphate buffer saline (PBS) (1:250), pH 7.4, and incubated overnight at 4 °C. After 2 washes with 400 μl/well of PBS containing 0.05% Tween-20 (PBST), the wells were blocked with 1% bovine serum albumin (BSA) in PBS and incubated at room temperature for 2 h. Thereafter 100 μl/well of the standards of known concentrations and each fecal extracts (diluted 1:500 in 1% BSA-PBST) were added and incubated at room temperature for 2 h. After washing, 100 μl/well diluted HRP-conjugated anti-mouse IgA monoclonal antibody (1:250) were added and incubated at room temperature for 1 h. Plates were washed again, 100 μl/well of 3,3′,5,5′-tetramethylbenzidine (TMB) substrate solution was added to each well, and incubated at room temperature for 15 min. The reaction was stopped by adding 100 μl of 2 M $H_2SO_4$ to each well to each well. Optical densities were measured at 450 nm and IgA concentrations were deter-mined from the reference curve. All samples were individually tested in duplicate.

**Table 1**
Sequences of primer used for RT-PCR.

| Gene | Primer sequence | Product size (bp) |
|---|---|---|
| HPRT | Forward: 5′-AAAAGCCAAATACAAAGCCTAAG-3′<br>Reverse: 5′-ACGCAGCAACTGACATTTC-3′ | 182 |
| TGF-β | Forward: 5′-GGCGGTGCTCGCTTTGTA-3′<br>Reverse: 5′-GTTGTTGCGGTCCACCATTAG-3′ | 130 |
| IL-6 | Forward: 5′-ACAAGAAAGACAAAGCCAGAGT-3′<br>Reverse: 5′-TGCCGAGTAGATCTCAAAGTG-3′ | 229 |
| TNF-α | Forward: 5′-GGGCAGGTCTACTTTGGAG-3′<br>Reverse: 5′-CACTGTCCCAGCATCTTGT-3′ | 224 |

### 2.7. Quantification of target genes by real-time PCR

The total RNA extraction of intestinal tissues and reverse transcription were performed essentially as described previously (Yuan, Wu, Chen, Wu, & Hu, 2010). Relative quantitation of TGF-β, IL-6, TNF-α cDNA and HPRT (the house keeping gene) message were conducted on ABI 7300 (PE Applied Biosystems, USA). Amplification was carried out in a total volume of 25 μl containing 2.5 μl of 10× PCR buffer, 1.2 μl of $MgCl_2$ (25 mM), 2 μl of dNTP mix (2.5 mM), 0.3 μl of Tag DNA polymerase (5 U/μl), 2 μl of cDNA template, 2 μl (5 μM) of each target gene and HPRT specific primers (Table 1), 1.25 μl of 20× EvaGreen™ dye, 0.5 μl of 50× ROX reference dye. Reaction was performed for pre-denaturation at 95 °C for 2 min, denaturation step of 5 s at 95 °C, annealing and elongation at 60 °C for 31 s with 40 PCR cycles, and then the dissociation curve was collected with one cycle of 95 °C 15 s, 60 °C 1 min, 95 °C 15 s and 60 °C 15 s. Relative quantification between samples was achieved by the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001) and is reported as the n-fold difference relative to target gene mRNA expression in the calibrator group (the group of mice orally administered with saline).

### 2.8. Histological examination for intraepithelial lymphocyte (IEL)

The fixed tissue samples were embedded in paraffin and serially sectioned at a thickness of 6 μm. After hematoxylin–eosin (H.E.) staining, the sections were sealed with a coverslip. Epithelial cells and lymphocytes at five different fields of intestinal villi in each mouse were counted for the statistical analysis of the data.

### 2.9. Immunohistochemical staining for $IgA^+$ cells

The fixed samples of duodenum were embedded in paraffin, serially cut into 6 μm-thicknesses and mounted on polylysine-coated glass slides. The sections were immunostained by the indirect immunoperoxidase method (Khan, Hashimoto, Iwami, & Iwanaga, 1997). Briefly, endogenous peroxidase was inhibited with 3% $H_2O_2$ in methanol for 10 min, and then rinsed in PBS for 5 min 3 times. The sections were incubated in 0.01 M citrate-buffered solution (pH 6.0) at 95 °C for 20 min for antigen retrieval, then allowed to cool at room temperature and rinsed in PBS 3 times. After that,

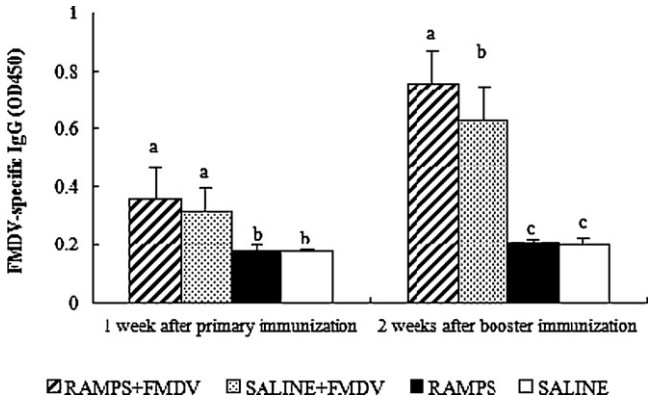


**Fig. 1.** FMDV-specific IgG responses. Bars with different letters are statistically different ($P < 0.05$).

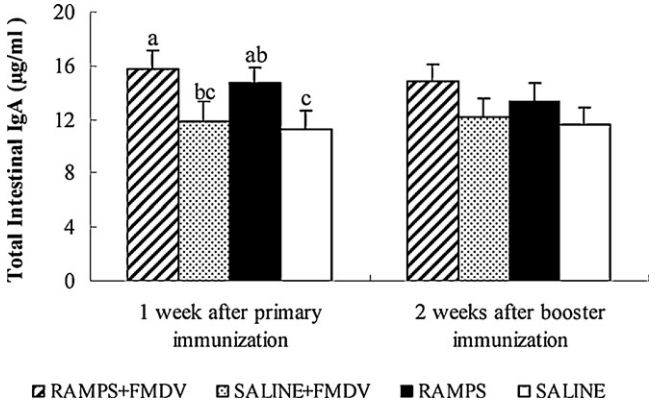


**Fig. 2.** Total intestinal IgA levels in the duodenum. Bars with different letters are statistically different ($P < 0.05$).

the sections were treated with 5% goat serum in PBS to block non-specific binding at room temperature for 30 min. The excessive solution was shaken off and the slides around the tissue section were blotted. The sections were incubated with rabbit anti-mouse IgA antibody (1:800) at 4 °C over night, and then washed three times in PBS for 15 min, followed by incubation with goat anti-rabbit IgG conjugated HRP (1:500) at 37 °C for 1 h. After the sections were rinsed with PBS for 15 min, the reactions were made visible with metal-enhanced diaminobenzidine (DAB) (BOSTER, China). All incubations were performed in a moist chamber. Control staining was carried out simultaneously in which the first antibody was replaced with PBS. No specific staining was found in the control.

### 2.10. Statistical analyses

The sections were observed at a light microscope (Nikon, Japan). Five different regions were randomly selected in each section and the images were captured with a video camera connected to a computer. The number of IELs per 100 epithelial cells and the relative area of $IgA^+$ cells in five different microscope fields of intestinal villi were counted using Nikon NIS element BR 2.30 software (Nikon, Japan).

Data were expressed as means ± standard deviations (S.D.). Duncan's test was used to compare the parameters between groups by using SPSS 13.0. $P$ values of less than 0.05 were considered statistically significant.

## 3. Results

### 3.1. Serum FMDV-specific IgG levels

To determine the effect of oral administration of RAMPS on the specific antibody responses, mice were orally administered saline or RAMPS at a dose of 0.05 g for 4 days, and then immunized SC twice with 200 μl of FMDV vaccine at 2-week intervals. Blood samples were collected for measurement of FMDV-specific IgG levels. Fig. 1 indicates that RAMPS elicited significantly higher IgG responses two weeks after the booster immunization ($P < 0.05$) but not one week after the first immunization when compared to the control.

### 3.2. Effect of oral administration of RAMPS on intestinal total IgA

Fig. 2 shows that the total sIgA concentration of the feces in the duodenum significantly increased ($P < 0.05$) one week but only numerically increased two weeks after the end of administration of RAMPS when compared to the control.

### 3.3. Effect of oral administration of RAMPS on $IgA^+$ cells in duodenum

As shown in Fig. 3, IgA secreting cells in the lamina propria of villi in the duodenum were round and had a nucleus surrounded by a ring of yellow–brown cytoplasm. Fig. 4 shows that the areas of $IgA^+$ cells in the duodenum significantly increased ($P < 0.05$) one week but only numerically increased two weeks after the end of administration of RAMPS when compared to the control.

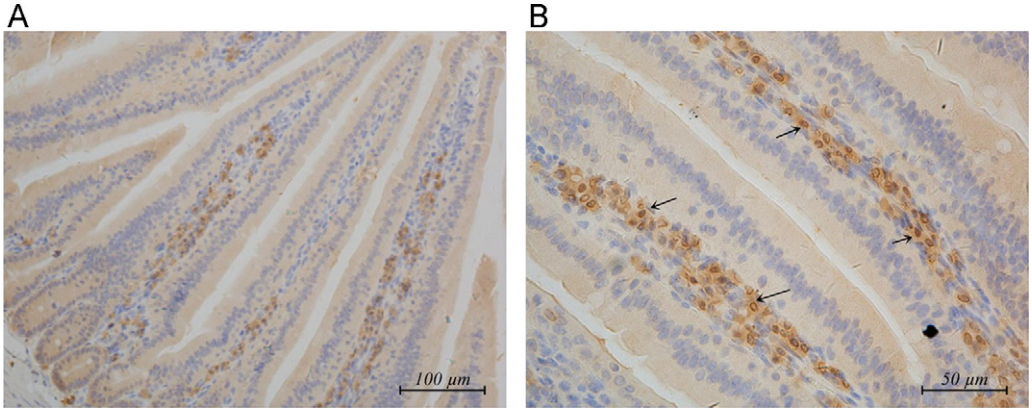


**Fig. 3.** IgA-positive cells (→) in the duodenum are round or ellipse, with a nucleus surrounded by a ring of yellow–brown cytoplasma. (A) 200× and (B) 400×. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

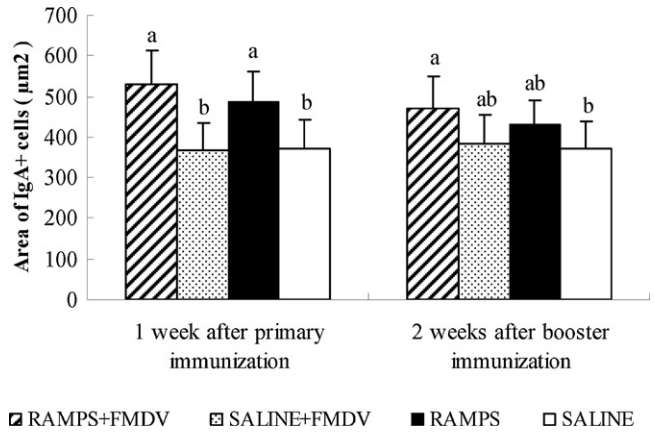


**Fig. 4.** The areas ($\mu m^2$) of IgA-positive cells in the duodenum. Bars with different letters are statistically different ($P < 0.05$).

### 3.4. Effect of oral administration of RAMPS on cytokine mRNA expression in duodenum

Fig. 5 shows that mRNA expressions of TGF-β, IL-6 and TNF-α in the duodenum significantly increased ($P < 0.05$) one week but only numerically increased two weeks after the end of administration of RAMPS when compared to the control.

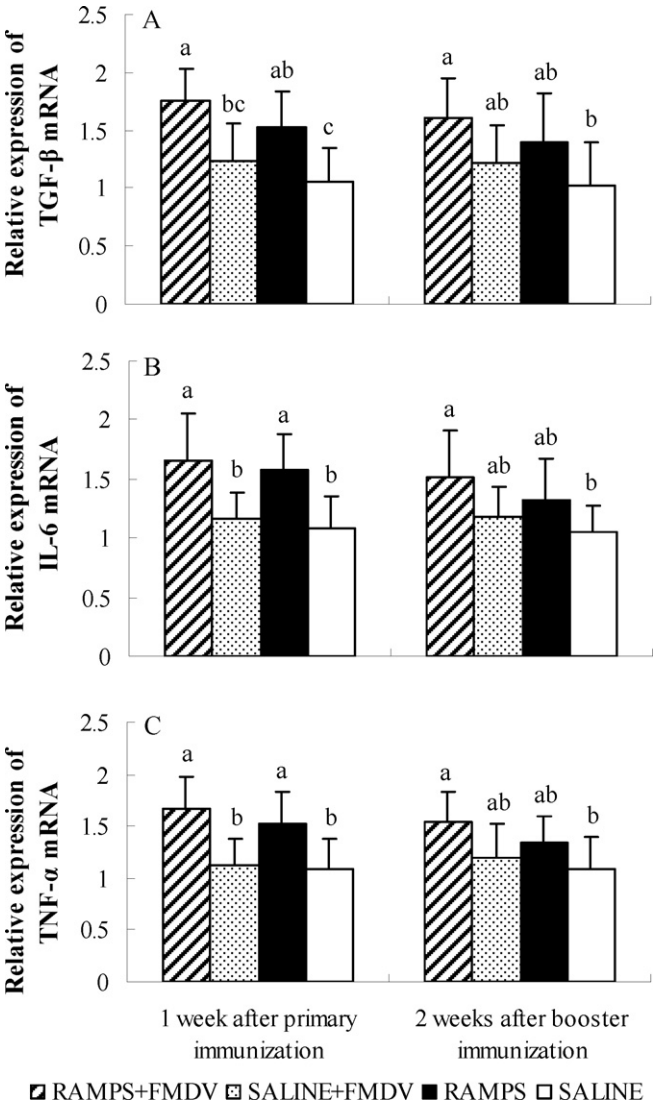


**Fig. 5.** Cytokine mRNA expression in the duodenum. Bars with different letters are statistically different ($P < 0.05$).

### 3.5. Effect of oral administration of RAMPS on IELs in duodenum

As shown in Fig. 6, the IELs were dispersed as single cells within the epithelial cell layer that surrounds the intestinal lumen in the basal region of the epithelium in duodenum. Fig. 7 shows that the number of IELs in the duodenum significantly increased ($P < 0.05$) one week but only numerically increased two weeks after the end of administration of RAMPS when compared to the control.

## 4. Discussion

We previously demonstrated that oral administration of an herbal liquid (RAM) or polysaccharide (RAMPS) made from the rhizome of *Atractylodis macrocephalae Koidz.* significantly increased the immune response to FMD vaccination (Li et al., 2009; Xie et al., 2012). In this study, we demonstrated that an increased serum IgG response to FMD vaccine was associated with the enhanced gut mucosal immunity by RAMPS. The mice orally administered RAMPS for 4 days had significantly enhanced both serum specific IgG response and improved gut mucosal immunity as indicated by elevated intestinal total IgA, mRNA expression of TGF-β, IL-6 and TNF-α, the area of $IgA^+$ cells and the number of IELs in duodenum.

Polysaccharides contained in the medicinal herbs such as *Radix ginseng*, *Radix astragaliseu hedysari*, *Rhizome atractylodis macrocephalae* have been reported to have immunostimulating activities (Lim, Na, Choi, Chung, & Hwang, 2004; Xie et al., 2012; Zhang et al., 2010). Since the traditional herbal medicines are administered through the oral route, the polysaccharides in herbs are difficult to be absorbed into blood circulation by the gastrointestinal tract because of their high molecular weight. It is possible for the polysaccharides directly act on the enteric mucosa, and activate the enteric mucosal immune system. Secretory IgA (sIgA) from B cells in the laminal propria of the intestine is the important effector molecules to protect mucosal surfaces. It has the mechanisms for antigen neutralization, prevention of microbial attachment to epithelial surface, elimination of excessive antigen load, and the overall maintenance of mucosal homeostasis (Johansen et al., 1999; Lamm, 1997; Uren et al., 2003). IgA secreting cells are often used to evaluate the intestinal mucosal immunity (Zhang, Zhang, & Yang, 2007). As shown in Fig. 2, one week after oral administration of RAMPS, total sIgA in the duodenum significantly increased ($P < 0.05$) in both immunized and unimmunized mice when compared to the control, indicating that the intestinal mucosal immunity was enhanced by RAMPS. Enhanced production of total sIgA may be explained by increased $IgA^+$ cells as shown in Fig. 4. Increased $IgA^+$ cells by herb-derived polysaccharides have been reported by others. Liu, Zhou, Wang, and Hu (2000) reported that $IgA^+$ cells were significantly increased in the small intestine of mice after oral administration of polysaccharides extracted from Sijunzi decoction (consisting of *Radix ginseng*, *Rhizome atractylodis macrocephalae*, *Poria* and *Radix glycyrrhizae*). Zhou et al. (2009) observed that a polysaccharide extract of *Ganoderma lucidum* significantly enhanced the number of $IgA^+$ cells in small intestine in $H_{22}$ liver cancer-bearing mice.

TGF-β signaling plays a central role for the induction of mucosal IgA. In vitro tests showed that TGF-β stimulates the isotype switch to IgA, and IgA secretion by LPS-stimulated mIgA-B cells from Peyer's patches and spleen (Ehrhardt, Strober, & Harriman, 1992; Kim & Kagnoff, 1990). Further vivo studies indicated that IgA responses are suppressed in TGF-β1 knockout mice (van Ginkel et al., 1999) or mice lacking the TGF-β receptor in B cells (Cazac & Roes, 2000). IL-6 is important in regulating the effector stage

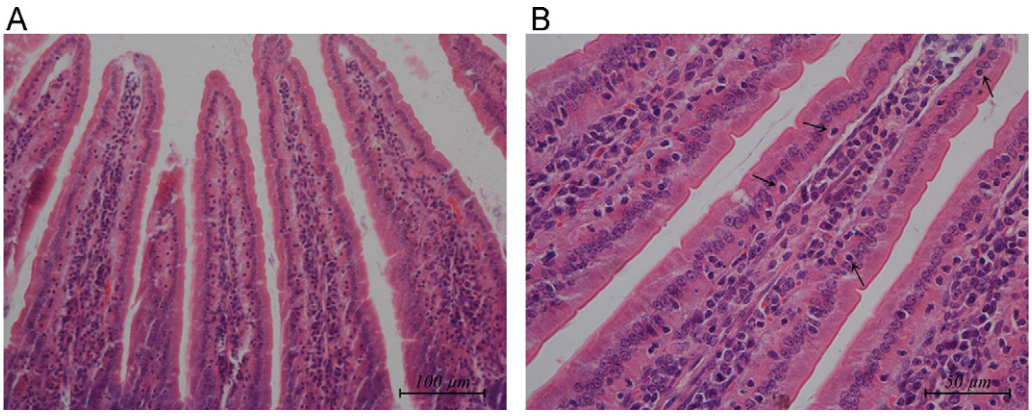


**Fig. 6.** Intraepithelial lymphocytes (IELs) (→) in the duodenum are dispersed as single cells within the epithelial cell layer. (A) 200× and (B) 400×.

of IgA responses, which markedly and selectively enhances IgA production in vitro by isotype-committed B cells (Beagley et al., 1989). IL-6 is also critical for the development of mucosal antibody responses in vivo. This is probably the actions of locally produced IL-6 in promoting differentiation or proliferation of plasma cell precursors arriving at mucosal sites (Ramsay et al., 1994). So the increased production of sIgA and $IgA^+$ cells may be due to up-regulated mRNA expression of TGF-β and IL-6 in duodenum (Fig. 5). TNF-α is well recognized for its role in mediating innate immune responses (Trevejo et al., 2001). Some reports have found that TNF-α causes an increase in intestinal permeability (Gitter et al., 2000; Yan et al., 2006) and can be used as a mucosal adjuvant (Kayamuro et al., 2009). Fig. 5 shows that the mRNA expression of TNF-α in duodenum was increased by oral administration of RAMPS.

Intestinal intraepithelial lymphocytes (IELs) are immunocompetent cells of gut-associated lymphoid tissue (GALT) that the first encounter enteric pathogens entering the mucosa and located between intestinal epithelial cells (IEC) in the epithelium above the basement membrane (Yoshikai, 1999). It is well known that IELs are programmed for cytokine production to protect against bacterial and viral infections. It has been reported that the characteristic, distribution and the number of IELs rely on the mucosal immunization in different species, suggesting that IELs play an important role in gut mucosal immunity (Sim, 1995; Taguchi et al., 1991). In the present study, significantly increased IELs were found in the duodenum of mice orally administered RAMPS when compared to the control. The results suggest that RAMPS could stimulate enteric mucosal lymphocytes to regulate the immune response. Increased intestinal IELs by medicinal herbs have been found in other studies. Zhai, Li, Wang, Wang, and Hu (2011) reported that oral administration of GSLS solution significantly increased intestinal IELs in chickens immunized with ND live vaccine. Shi, Yan, Wang, Zhang, and Yu (2011) observed that IELs was also significantly increased in the small intestinal mucosa of stressed mice after oral administration with the water extraction from herbal formula consisting *Astragalus membranaceus*, *Ligustrum lucidum* and *Carthamus tinctorius* L.

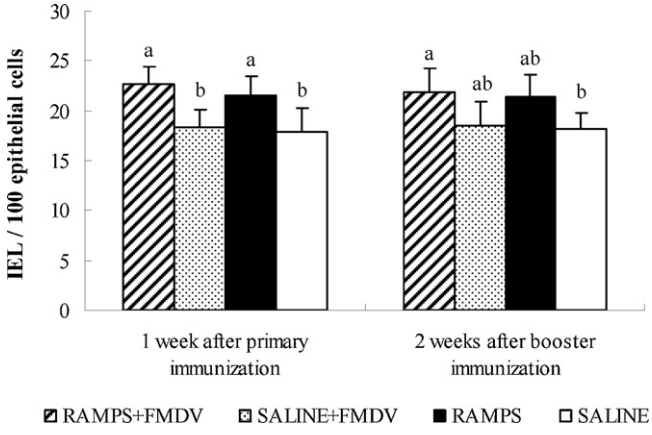


**Fig. 7.** The number of IELs per 100 epithelial cells in the duodenum. Bars with different letters are statistically different ($P < 0.05$).

Fig. 1 indicates that significantly increased serum IgG response to FMD vaccine took place two weeks after booster immunization. However, the significantly increased gut mucosal immunity was not found two weeks after booster immunization but one week after primary immunization. The reason might be that the effect on the mucosal immunity declined quickly after terminating oral administration of RAMPS, but it had triggered the innate immunity and bridged the adaptive immune response. After observing the above results, it is supposed that oral administration of RAMPS enhanced mucosal immunity in the intestinal tract, which then facilitated the systemic immune response to FMD vaccination. To effectively utilize RAMPS as an oral immunomodulator, the detailed mechanisms for the enhancement of the systemic immune responses by oral administration RAMPS remain to be further investigated.

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