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Adjuvant effect of *Atractylodis macrocephalae Koidz.* polysaccharides on the immune response to foot-and-mouth disease vaccine

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ABSTRACT

Present study was designed to investigate the polysaccharide (RAMPS) extracted from the rhizome of *Atractylodis macrocephalae Koidz.* (RAM) for its effect on the immune responses to foot-and-mouth (FMD) vaccine. Thirty-five ICR mice were randomly distributed into 5 groups with 7 mice in each. After oral administration of RAMPS for 4 days at a dose of 0, 0.025, 0.05, 0.1 g or 0.25 g of RAM, respectively, the animals were immunized twice with FMD vaccine at 2-week intervals. Three weeks later, serum IgG, IFN-γ/IL-5, splenocyte proliferation, and mRNA expression of cytokines by splenocytes were measured. Results indicated that RAMPS and RAM significantly enhanced IgG titers, IgG subclasses, IFN-γ, IL-5 and the splenocyte proliferations stimulated by concanavalin A, lipopolysacharide, and FMDV ($P < 0.05$). Therefore, RAMPS and RAM increased both cellular and humoral immune responses to vaccination, and may have a potential as an oral adjuvant to improve vaccination.

1. Introduction

Foot-and-mouth disease (FMD) is a highly contagious disease affecting cloven-hoofed animals such as cattle, swine and sheep. It is one of the most important animal diseases and may exert destructive socio-economic impacts on the countries affected (Mayr, O'Donnell, Chinsangaram, Mason, & Grubman, 2001). For example, the 2010 FMD outbreak in Japan and South Korea caused billions of dollars of losses (Rome, 2010). Vaccination with inactivated FMD virus (FMDV) is a major means for the control of this disease in many countries. However, this inactivated FMD vaccines do not induce broadly reactive long-term protection (Rodriguez & Grubman, 2009), and failure to elicit effective immune responses by vaccination in some areas in China has been reported. For examples, Tian and He (2008) analyzed 129 serum samples of the pigs having received vaccination against FMD and found that only 54.2% of the samples had antibody titers required for immune protection immunity. Liu, Yang, and Wang (2010) observed only 51.33% of the pigs had produced immune responses with antibody titer high enough for protection following vaccination against FMD in Liping county of Guizhou Province of China. Therefore, searching for new approaches to the improvement of FMD vaccination is needed.

Strategies to improve the immune response to vaccination have included the use of higher vaccine dose or increasing number of doses, use of different administration route (e.g., intradermal versus intramuscular administration), accelerating dosing schedule and use of adjuvants such as antigen delivery systems and various immunomodulators (Bryan, Sjogren, Perine, & Legters, 1992; Disis et al., 1996; Mitwalli, 1996; Rey et al., 2000). There is growing evidence that medicinal herbs and the ingredients enhance the immune response to vaccination against infectious agents (Rajput, Hu, Xiao, & Arijo, 2007; Song, Bao, Wu, & Hu, 2009; Sun, Ye, Pan, & Pan, 2004; Yang, Sun, & Fang, 2005). *Atractylodis macrocephalae Koidz.* is a plant in family of Compositae with a plenty of natural resource in Zhejiang Provinces in China. The rhizome of the plant (RAM) has been utilized for at least 2000 years as a traditional Chinese medicine (Chen, He, Jiang, & Qiu, 2007). RAM contains different active ingredients, such as polysaccharides, volatile oil and lactones (Duan, Xu, Liu, & Li, 2008). Our previous study has demonstrated that oral administration of a decoction made from RAM has significantly increased immune responses to vaccines against FMD in mice (Li, Sakwiwatkul, Yutao, & Hu, 2009). But it is not clear that which ingredient(s) contribute the adjuvant activity. In this study, polysaccharide (RAMPS) extracted from RAM was investigated for its effect on the cellular and humoral immune responses to a commercial FMD vaccine in mice.

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.

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(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

Concanavalin A (ConA), lipopolysaccharide (LPS from *Escherichia coli* 055:B5) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co., USA; goat anti-mouse IgG peroxidase conjugate was from Kirkegaard & Perry Laboratories, Inc., USA; goat anti-mouse peroxidase conjugate IgG1, IgG2a, IgG2b and IgG3 were from Southern Biotech. Assoc., Birmingham, AL, USA; RNAiso^{TM} plus was from TaKaRa Biotechnology (Dalian) Co., Ltd., China; revert AidTM M-MuLV reverse transcriptase was from Fermentas, USA; diethylpyrocarbonate (DEPC) and ribonuclease inhibitor was from Biobasic, Canada; oligo (dT)18 was from Sangon, China.

2.3. Preparation of RAM decoction and RAM polysaccharides (RAMPS)

Dried rhizome of *Atractylodis macrocephalae Koidz.* (RAM) was purchased from Hu Qing Yu Tang Co. Ltd., Hangzhou, China. The drug source was in Pan'an County of Zhejiang Province of China where the best RAM is produced. RAM decoction was prepared according to method as previously described by Li, Sakwiwatkul, et al. (2009). Polysaccharide was extracted from RAM as follows. Briefly, the rhizome (100 g) was ground into powder and then extracted with boiling water two times under reflux for 2 h each time. The aqueous portion was filtered through filter paper. The filtrate was concentrated under reduced pressure, and then centrifuged at 3000 rpm for 15 min. Four volumes of 95% ethanol were added to the supernatant, and kept overnight at 4 °C. The resulting precipitate was dissolved in distilled water, subjected to Macroporous Adsorption Resin column D101, and then washed with water. The collected elute was concentrated, dialyzed against distilled water (cut-off Mw 7000 Da) and lyophilized to afford a total RAM polysaccharide (RAMPS, light off-white powder, 4.2 g).

Total sugar content was estimated by the phenol–sulfuric acid analysis using glucose as a standard (Bitter & Muir, 1962). Neutral monosaccharide composition was analyzed according to the following procedure: RAMPS (10 mg) were hydrolyzed with 5 ml of 2 M TFA at 110 °C for 6 h to release component monosaccharides. The hydrolyzed monosaccharides (inositol as the internal standard) were derivatized to acetylated aldononitriles (Mawhinney, Feather, Barbero, & Martinez, 1980) and isothermally separated by gas chromatography (GC) in an Agilent 6890N system (Agilent Technologies, Palo Alto, CA, USA) equipped with a flame-ionization detector (FID) and a DB-5 capillary column (30.0 m × 0.32 nm × 0.25 μm).

2.4. Immunization

Thirty-five female ICR mice were randomly divided into five groups with 7 mice in each. The animals were subcutaneously injected twice with 200 μl of FMDV type O vaccine (Lanzhou Veterinary Research Institute, China) with 2-week intervals. One day before each immunization, the mice had already been orally administered for 4 days with 0.25 ml of 0.89% saline solution, or RAMPS (0.025, 0.05 or 0.1 g) or RAM decoction (0.25 g). Blood samples were collected 3 weeks after the booster immunization for detection of IgG titers, the IgG subclasses and western blot analysis. Splenocytes were harvested for determination of lymphocyte proliferation and cytokines mRNA expression.

2.5. FMDV-specific IgG and the IgG subclasses

Serum samples were analyzed for measurement of serum IgG and the isotypes by an indirect double antibody sandwich enzyme-linked immunosorbent assay. The wells of polyvinyl 96-well microtiter plates were coated with 50 μl rabbit anti-FMDV serotype O antibody (LVRI, China) diluted in 0.05 M carbonate/bicarbonate buffer (1:800), pH 9.6, and incubated overnight at 4 °C. After five washes with phosphate buffer saline containing 0.05% Tween-20 (PBST), the wells were blocked with 5% skimmed milk and incubated at 37 °C for 2 h. Thereafter, 50 μl FMDV type O antigen (LVRI) (1:8 dilution) was added and incubated at 4 °C for 2 h. After washing, 50 μl of serum (diluted serially for IgG or diluted 1:50 for isotype analysis in PBS 5% skimmed milk) was added to each well and incubated at 37 °C for 1 h. Plates were then washed five times in PBST. For IgG titer detection, 50 μl of goat anti-mouse IgG horseradish peroxidase conjugate (1:5000) was added to the wells and incubated at 37 °C for 1 h. Plates were washed again with PBST. Fifty microliters of 3,3′,5,5′-tetramethyl benzidine solution (100 μg/ml of 0.1 M citrate–phosphate, pH 5.0) was added to each well and incubated for 15 min at 37 °C. The reaction was stopped by adding 50 μl of 2 M H_2SO_4 to each well. The optical density of the plate was read by an automatic ELISA plate reader at 450 nm. Values above the cut-off background level (mean value of sera from unimmunized mice multiplied by a factor of 2.1) were considered positive. Titers were depicted as reciprocal end-dilutions. For subclasses, 50 μl of horseradish peroxidase conjugated goat anti-mouse IgG1 or IgG2a or IgG2b or IgG3 (1:2000) was added to corresponding plate and then incubated for 1 h at 37 °C.

2.6. Splenocyte proliferation assay

Spleen was collected 3 weeks after the booster immunization from the immunized mice under aseptic conditions, and kept in Hank's balanced salt solution (HBSS, Sigma). The organ was minced and passed through a fine steel mesh to obtain a homogeneous cell suspension. To lyse contaminated erythrocytes, 0.83% NH_4Cl in 0.01 M Tris–HCl (pH 7.2) was added to the suspension. After centrifugation (380 × *g* at 4 °C for 10 min), the pelleted cells were washed three times in HBSS and re-suspended in complete medium (RPMI 1640 supplemented with 0.05 mM 2-mercaptoethanol, 100 IU/ml penicillin, 100 μg/ml streptomycin and 10% heat inactivated FBS). Cell numbers were counted with a haemocytometer by trypan blue dye exclusion method. Cell viability exceeded 95%. Splenocyte proliferation was assayed as described previously with some modification (Sun et al., 2004). Briefly, splenocytes were seeded into a 96-well flat-bottom microtiter plate (Nunc) at 5.0×10^6 cells/ml in 100 μl complete medium, thereafter Con A (final concentration 5 μg/ml), LPS (final concentration 7.5 μg/ml), FMDV antigen (final concentration 200 μg/ml) or medium were added giving a final volume of 200 μl. The plates were incubated at 37 °C in a humid atmosphere with 5% CO_2 for 2 or 5 days. All the tests were carried out in triplicate. The cell proliferation was evaluated using MTT method. Briefly, 50 μl of MTT solution (2 mg/ml) were added to each well 4 h before the end of incubation. The plates were centrifuged (1400 × *g*, 5 min) and the untransformed MTT was removed carefully by pipetting. To each well 150 μl of a DMSO working solution (192 μl DMSO with 8 μl 1 M HCl) was added, and the absorbance was evaluated in an ELISA reader at 570 nm with a 630 nm reference after 15 min. The stimulation index (SI) was calculated based on the following formula: SI = the absorbance value for mitogen cultures/the absorbance value for non-stimulated cultures.

2.7. Quantification of target genes by real-time PCR

Splenocytes from the FMDV-immunized ICR mice prepared as described previously were seeded into a 24-well flat-bottom microtiter plate (Nunc) at 5×10^6 in 2 ml complete medium, thereafter 100 μl FMDV antigen (2 μg/μl) was added. The plates were incubated at 37 °C in a humid atmosphere with 5% CO_2. After 15 h treatment, cells were harvested by centrifugation ($380 \times g$ at 4 °C for 10 min), and washed with ice-cold PBS, then subjected to RNA extraction. Splenocytes (1×10^7) were lysed in 1 ml of RNAiso^{TM} Plus reagent and the total RNA was isolated according to the manufacture's protocol. The concentration of total RNA was quantified by determining the optical density at 260 nm. The total RNA was used and reverse transcription was performed by mixing 2 μg of RNA with 0.5 μg oligo $(dT)_{18}$ primer in a sterile tube. Nuclease-free water was added giving a final volume of 12.5 μl. This mix was incubated at 70 °C for 5 min and chilled on ice for 2 min. Then a solution containing 4 μl of M-MuLV 5× reaction buffer, 2 μl of 10 mM dNTP, 20 U of ribonuclease inhibitor, and DEPC-treated water was added giving a final volume of 19 μl and the tubes were incubated for 5 min at 37 °C. The tubes then received 200 U of M-MuLV reverse transcriptase and were incubated for 60 min at 42 °C. Finally, the reaction was stopped by heating at 70 °C for 10 min. The samples were stored at −20 °C until further use.

Relative quantitation of GATA-3, T-bet and cytokines cDNA and β-actin message was conducted on ABI 7300 (PE Applied Biosystems, USA). The purpose of the house keeping gene (β-actin) is to normalize the PCRs for the RNA added to the reverse transcription reactions and to correct for differences in RT reaction efficiencies (Bustin, 2000). The PCR was performed in a dual PCR with primers and TagMan probes for the target gene and β-actin cDNA in the same reaction vessel. The primers and probes for target gene and β-actin (Table 1) were designed using Primer Express 3.0 (PE Applied Biosystems) according to the manufacturer's directions and each primer was located in different exons of target genes. Probes for GATA-3, T-bet and cytokines were detected via a 5′ labeled with reporter dye (FAM) (Sangon Co., Ltd., Shanghai, China) and 3′ labeled with quench dye (BHQ-1) (Sangon Co., Ltd., Shanghai, China), while probes for β-actin were 5′ labeled with reporter dye (HEX) (Sangon Co., Ltd., Shanghai, China). The PCR products were identified by DNA sequencing.

Amplification was carried out in a total volume of 20 μl containing 2 μl of 10× PCR buffer, 2 μl of $MgCl_2$ (25 mmol/L), 2 μl of dNTPmix (2.5 mM), 0.4 μl of Tag DNA polymerase (Takara, China), 2 μl of cDNA template, 2 μl (5 μM) of each target gene and β-actin specific primers, 1 μl (5 μM) of target gene and β-actin specific probes. Reaction conditions were the standard conditions for the TagMan PCR (15 s denaturation at 95 °C, 30 s annealing at 60 °C) with 40 PCR cycles. Relative quantification between samples was achieved by the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001) and calculated by software REST 2005 (gifted by Eppendorf Company), 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).

Table 1
Sequences of primer and probes for quantitative RT-PCR of cytokine and transcription factors.

Gene	Primer sequence
β-Actin	Forward: 5′-AGCGGTTCCGATGCCCT-3′ Reverse: 5′-AGAGGTCTTTACGGATGTCAACG-3′ Probe: 5′-*HEX*-TCCTTCTTGGGTATGGAATCCTGTGGC-*BHQ-1*-3′
IL-4	Forward: 5′-GAGACTCTTTCGGGCTTTTCG-3′ Reverse: 5′-CAGGAAGTCTTTCAGTGATGTGG-3′ Probe: 5′-*FAM*-CCTGGATTCATCGATAAGCTGCACC-*BHQ-1*-3′
IFN-γ	Forward: 5′-GCTTTGCAGCTCTTCCTCATG-3′ Reverse: 5′-CTTCCACATCTATGCCACTTGAG-3′ Probe: 5′-*FAM*-CTGTTTCTGGCTGTTACTGCCACGGC-*BHQ-1*-3′
GATA-3	Forward: 5′-GGTCAAGGCAACCACGTC-3′ Reverse: 5′-CATCCAGCCAGGGCAGAG-3′ Probe: 5′-*FAM*-CGCCCGCCTCTGCTGCACG-*BHQ-1*-3′
T-bet	Forward: 5′-ATTGCCCGCGGGGTTG-3′ Reverse: 5′-GACAGGAATGGGAACATTCGC-3′ Probe: 5′-*FAM*-CTGGGAAGCTGAGAGTCGCGCTCA-*BHQ-1*-3′

2.8. Determination of serum cytokines by western blot

Serum samples were analyzed for measurement of IFN-γ and IL-5 by Western blot analysis. For the determination of IFN-γ, the serum samples were diluted with the sample buffer (1:50), boiled for 5 min, and then 10 μl of each diluted sample was separated by 12% sodiumdodecyl sulfate-polyacrylamide gelelectrophoresis (SDS-PAGE). The proteins were transferred onto the immobilon-p transfer membrane (Millipore Corporation, USA), the membrane was washed and then blocked in 5% (skimmed milk in TBS) blocking agent for 35 min at 37 °C in the incubator shaker. After three washes in TBST (TBS containing 0.1% Tween-20), the membrane was incubated with 1:1000 diluted IFN-γ monoclonal antibody (sc-5992, Santa Cruz Biotechnology Inc., SantaCruz, CA, USA) for 50 min at room temperature on the rocker platform. The membrane was washed three times in TBST, followed by incubating with HRP-conjugated goat anti-mouse IgG at a 1:2000 dilution for 50 min at room temperature. After the final wash, the immunoblot was examined by BeyoECL Plus (Beyotime Biotechnology, China) according to the manufacturer's instructions. The membrane was exposed to an X-ray film, which was later developed. For determination of IL-5, the serum samples were at a 1:20 dilution, and incubated with 1:800 diluted IL-5 polyclonal antibody (BS3471, Bioworld Technology, Inc., USA), followed by the corresponding secondary HRP-conjugated goat anti-rabbit IgG (Proteintech Group Inc., USA) at a 1:2000 dilution, The other procedures was the same as described above for the detection of IFN-γ. The intensity of the resulting bands was quantified by the Quantity One® 1-D Analysis Software Version 4.6.2 (BioRad, Hercules, CA, USA).

2.9. Statistical analyses

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. Isolation, characterization and composition of RAMPS

The polysaccharide contained in RAMPS was 86.2% as determined by phenol–sulfuric acid method. RAMPS showed negative Fehling's reagent and iodine–potassium iodide reactions, indicating that it did not contain reducing sugar and starch-type polysaccharides. UV analysis and triketohydrindene hydrate reaction indicated that RAMPS was not contaminated with proteins. GC quantitative analysis with derivatization revealed that RAMPS 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.

Table 2
Effects of oral administration of RAM and RAMPS on the mean body weight (mean ± S.D. g) of mice ($n = 7$).

Group/time		Before immunization*	Before booster*	3 weeks after booster*
Saline		25.05 ± 1.12	29.90 ± 1.66	34.26 ± 1.81
RAM	0.25 g	25.12 ± 1.15	29.99 ± 2.18	34.26 ± 2.53
RAMPS	0.025 g	23.97 ± 1.06	29.19 ± 1.68	33.85 ± 2.21
	0.05 g	24.71 ± 1.11	29.04 ± 1.52	34.05 ± 2.40
	0.1 g	24.64 ± 1.33	30.01 ± 2.30	34.94 ± 2.44

* No statistical difference between groups at the same time ($P > 0.05$).

3.2. Effects of oral administration of RAMPS and RAM on the body weight of mice

To determine if RAMPS has the negative effect on the growth performance of mice, the body weight of each animal was measured before and after immunization. No abnormal behavior and side effects were found in mice throughout the experiment. There was no significant difference for the body weight between the mice administered RAM or RAMPS and the control mice administered saline solution as indicated in Table 2 ($P > 0.05$).

3.3. FMDV-specific IgG and IgG isotypes

To determine the effect of oral administration of RAMPS on the specific antibody responses, mice were orally administered saline (control) or RAM or RAMPS for 4 days, and then immunized s.c. twice with 200 μl of FMDV vaccine at 2-week intervals. Blood samples were collected 3 weeks after boosting for measurement of FMDV-specific IgG titers and the IgG subclasses. Fig. 1 indicates that FMDV-specific IgG titers were higher in mice orally administered RAM or RAMPS than in mice administered saline solution only ($P < 0.05$). Moreover, mice administered RAMPS at a dose of 0.05 g had almost 10-fold higher FMDV-specific IgG titers (1:2826) than the control (1:290) ($P < 0.05$).

Fig. 2 shows that the subclasses IgG1, IgG2a, IgG2b and IgG3 levels were higher in groups administered RAM or RAMPS than in the control group ($P < 0.05$). RAMPS at a dose of 0.05 g elicited significantly higher IgG1, IgG2a, IgG2b ($P < 0.05$) but not IgG3 responses compared to RAM.

3.4. Effect of RAMPS and RAM on splenocyte proliferation

Effects of RAMPS on mitogen- and FMDV-stimulated splenocyte proliferation in the immunized mice are shown in Fig. 3. When compared with the control, RAM or RAMPS significantly enhanced splenocyte proliferative responses to ConA, LPS, or FMDV ($P < 0.05$). Among groups, mice administered RAMPS at a dose of 0.05 g were found to have numerically highest splenocyte proliferation.

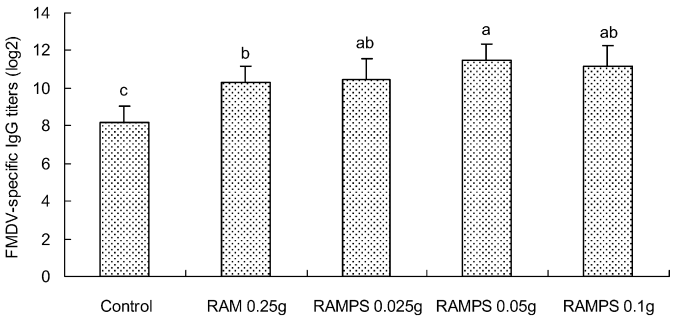

Fig. 1. FMDV-specific IgG titers. Bars with different letters are statistically different ($P < 0.05$).

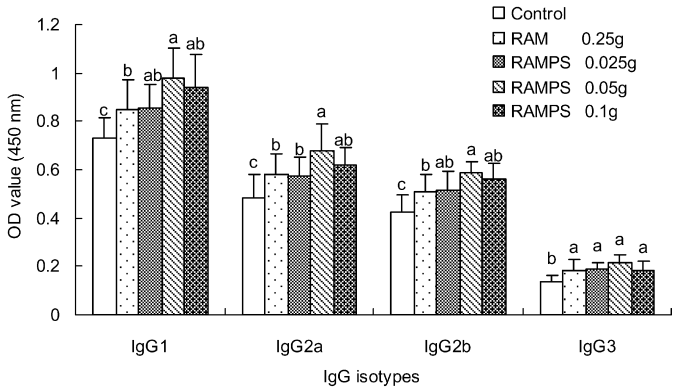

Fig. 2. FMDV-specific IgG isotypes. Bars with different letters are statistically different ($P < 0.05$).

3.5. Effect of RAMPS and RAM on mRNA expression of cytokines and transcription factors by splenocytes

Oral administration of RAM and RAMPS significantly increased mRNA expressions of cytokines IFN-γ and IL-4, and transcription factors T-bet and GATA-3 ($P < 0.05$) by splenocytes when compared with the control (Figs. 4 and 5). The rates of increase for Th1 response IFN-γ and T-bet were 2.34 (4.45/1.76) and 2.13 (3.57/1.68), respectively, which were numerically higher than those of Th2 response where 1.74 (2.53/1.46) for IL-4 and 1.58 (2.22/1.4) for GATA-3 were detected.

3.6. Effect of RAMPS on serum IFN-γ and IL-5 levels

Western blot analysis showed that RAMPS at the dose of 0.05 g significantly increased both IFN-γ and IL-5 ($P < 0.05$) in serum of mice immunized with FMD vaccine (Fig. 6).

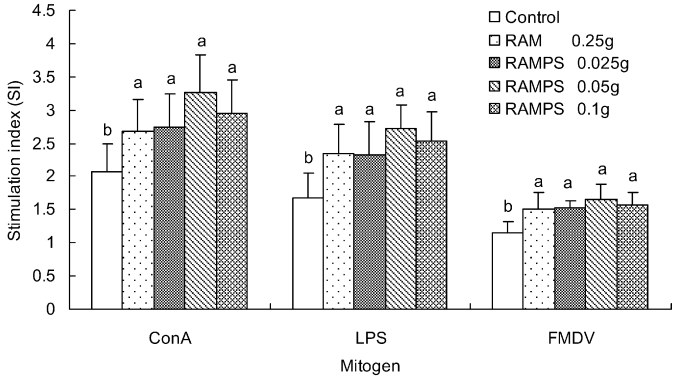

Fig. 3. Splenocyte proliferative responses to Con A, LPS and FMDV. Bars with different letters are statistically different ($P < 0.05$).

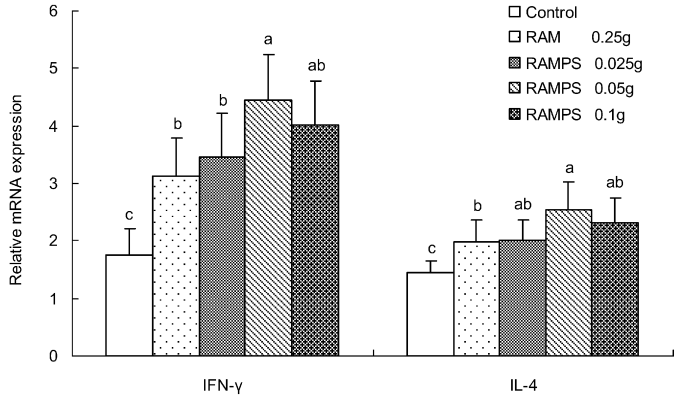

Fig. 4. Expression of IFN-γ and IL-4 mRNA by splenocytes. Bars with different letters are statistically different ($P < 0.05$).

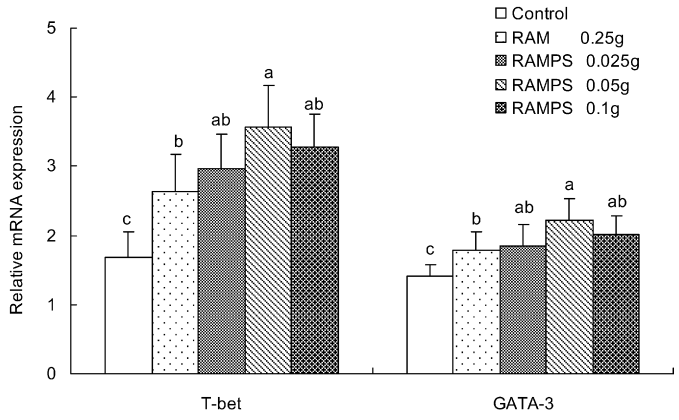

Fig. 5. Expression of T-bet and GATA-3 mRNA by splenocytes. Bars with different letters are statistically different ($P < 0.05$).

4. Discussion

In this study, oral adjuvant effect of RAMPS against FMD vaccine in mice has been proved. After oral administration for 4 days of RAMPS, immunization of a commercial FMD vaccine induced significantly higher serum specific IgG and the IgG isotype responses in association with up-regulated serum IFN-γ and IL-5. In addition, RAMPS significantly increased splenocyte proliferative responses to ConA, LPS and FMDV, as well as mRNA expression of Th1/Th2 cytokines (IFN-γ/IL-4) and transcription factors (T-bet/GATA-3) by splenocytes.

The rhizome of *Atractylodis macrocephalae Koidz.* has long been used as a digestive stimulator in China. Chemically, it contains three categories including volatile oil, lactones and polysaccharides (Duan et al., 2008). Volatile oil fraction is rich in sesquiterpenes and acetylenic compounds, such as atractylon, atractylol, selina-4(14),7(11)-dien-8-one, β-Se-linene, aromaden-drene, etc. (Li, Wen, Cui, Zhang, & Dong, 2007). This fraction is believed to have antitumor activities. Zhang, Zhang, Shi, and Liu (2006) observed that volatile oil from RAM significantly suppressed growth of Ehrlich ascites tumor and extended the life-span of mice after the animals were peritoneally administered RAM for 7 days at a dose of 50 or 100 mg/kg. Lactone fraction contains atractylenolide I, II, III, IV with anti-inflammatory properties (Li, He, Dong, & Jin, 2007). Li, He, and Jin (2007) discovered that atractylenolides I and III inhibited the production of TNF-α and NO in peritoneal macrophages stimulated with LPS in a dose-dependent manner. However, neither volatile oil nor lactones have been found adjuvant properties in our experiments (data not shown). Therefore, the adjuvant activities of RAM may be attributed to the fraction of polysaccharide (RAMPS).

Many plant-derived polysaccharides have been found to have immunomodulatory activities, such as the polysaccharides isolated from *Astragalus membranaceus* (Li, Chen, Wang, Tian, & Zhang, 2009), *Actinidia eriantha* (Sun, Wang, Xu, & Ni, 2009), *Acanthopanax senticosus* (Shen et al., 1991), and *Angelica sinensis* (Yang, Jia, Meng, Wu, & Mei, 2006). RAMPS was composed of rhamnose, arabinose, xylose, mannose, glucose, and galactose with molar ratios of 1.00:2.49:2.07:4.94:11.33:1.35. RAMPS has a backbone constructed by glucose, and the branches constituted by rhamnose

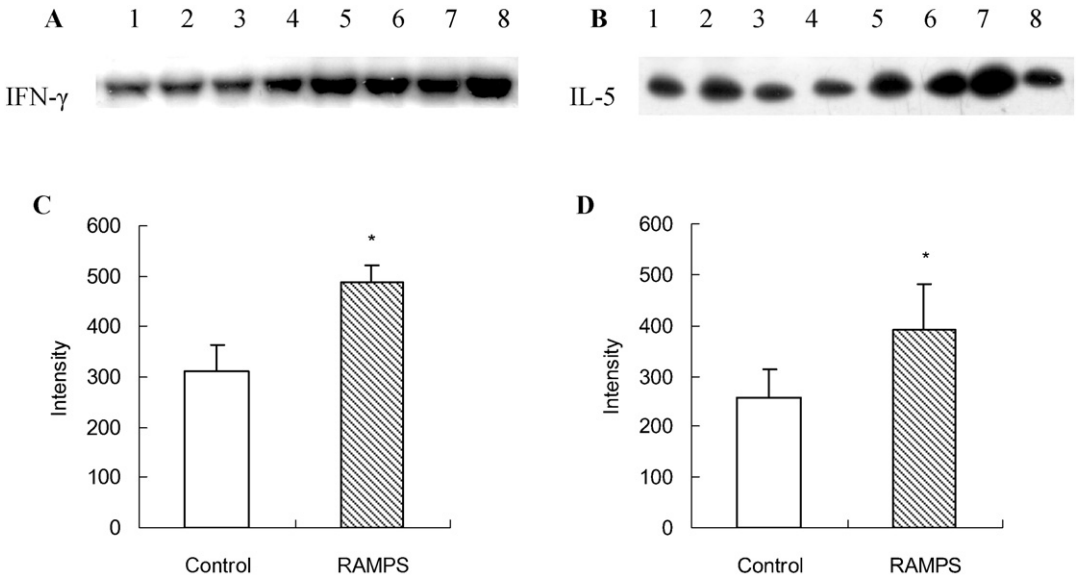

Fig. 6. Serum IFN-γ and IL-5 levels measured by western blot. Mice were orally administered saline (control) or RAMPS at a dose of 0.05 g for 4 days. (A) 10 μl of each diluted serum sample (1:50) was used for the determination of IFN-γ, lanes 1–4, control; lanes 5–8, RAMPS. (B) 10 μl of each diluted serum sample (1:20) was used for the determination of IL-5, lanes 1–4, control; lanes 5–8, RAMPS. IFN-γ (C) and IL-5 (D) were quantified by the Quantity One Software. *$P < 0.05$ between two groups.

and mannose (Liang, Guo, & Zhang, 2007). The polysaccharide isolated from the roots of *A. membranaceus* (APS) has been intensively studied for its immunomodulatory activities in China (Kang et al., 2010; Li, Chen, et al., 2009; Zhang et al., 2010). It contains rhamnose, glucose, galactose, arabinose with molar ratios of 1.19:72.01:5.85:20.95, and the main chain was composed of glucose (Li, Zhao, & Lv, 2008). Although RAMPS and APS have different sugar composition, many investigations have shown that polysaccharide structure has influence on biological activity more than the sugar composition (Deters, Lengsfeld, & Hensel, 2005; Sakagami et al., 2005; Zhao, Kan, Li, & Chen, 2005). Therefore, similar molecular structures of RAMPS and APS may contribute to their immunomodulatory effects.

In addition to promoting an appropriate immune response, an ideal adjuvant must be less toxic. Medicinal herbs are traditionally administered via oral route, and oral use of immunomodulators can avoid the side effects found in parenteral administration (Chavali & Campbell, 1987; Maharaj, Froh, & Campbell, 1986). As indicated in Table 2, there was no significant difference of the body weight between groups, and no abnormal behavior and side effects were found throughout the experiment. Therefore, RAMPS could be safe for oral administration.

The efficacy of vaccination against FMD is generally evaluated by humoral immune response (McCullough et al., 1986, 1992; Yadav, Sharma, & Chhabra, 2005). As shown in Fig. 1, oral administration of RAMPS significantly increased serum FMDV-specific IgG titers in mice, which indicated that RAMPS was able to improve humoral immunity when used as an adjuvant in FMD vaccine. Polysaccharide-enhanced humoral immune response has also been found in other study. Sun and Liu (2008) observed a significantly increased OVA-specific IgG levels in mice immunized with OVA in combination with polysaccharide derived from the mycelium of *Polyporus albicans*. Cellular immune response, mediated by T lymphocytes, plays an important role in the host response to intracellular pathogens. An increasing evidence suggested that T cell mediated immunity is required for protection against FMD in animals (Collen, Pullen, & Doel, 1989). The capacity to elicit an effective T cell immunity can be measured by lymphocyte proliferation response (Marciani et al., 2000). Fig. 3 shows that RAMPS significantly enhanced ConA-, LPS- and FMDV-stimulated splenocyte proliferation in the immunized mice, suggesting that both T and B cells were activated.

Immunity to different infectious agents requires distinct types of immune responses. Th1 immune response is required for protective immunity against intracellular infections, which is characterized by the production of cytokines IL-2, TNF-α and IFN-γ, and an enhanced production of IgG2a, IgG2b and IgG3 in mice. On the other hand, Th2 immunity is effective for protection against most bacterial as well as certain viral infection, which is characterized by the production of cytokines IL-4, IL-5 and IL-10, and an increased production of IgG1 (Constant & Bottomly, 1997; Del Prete, De Carli, Ricci, & Romagnani, 1991; Fiorentino, Bond, & Mosmann, 1989; Livingston et al., 1994). As shown in Fig. 2, RAMPS not only significant enhanced FMDV-specific IgG1 level in mice immunized with FMD vaccine, but also increased specific serum IgG2a, IgG2b and IgG3 levels, indicated that both Th1 and Th2 immune responses were activated. Similar results of plant polysaccharides have been reported by several workers (Lai et al., 2010; Sun & Liu, 2008; Sun et al., 2009). Sun et al. (2009) observed a significantly increased OVA-specific IgG1, IgG2a and IgG2b antibody titers in the immunized mice when OVA was administered subcutaneously with the water-soluble polysaccharide from the roots of *A. eriantha*. Lai et al. (2010) reported that a polysaccharide extract of *Ganoderma lucidum* induced significant levels of IgG1 and IgG2a antibodies specific for tetanus toxoid when immunized intramuscularly with tetanus toxoid in mice.

Enhanced production of all IgG subclasses may be explained by increased release of Th1 and Th2 cytokines. Fig. 4 shows that the mRNA expression of IFN-γ (Th1 cytokine) and IL-4 (Th2 cytokine) in splenocytes of the immunized mice were both enhanced by RAMPS, which may be associated with simultaneously up-regulated mRNA expression of T-bet and GATA-3 in splenocytes (Fig. 5). Furthermore, a significant increase of IFN-γ and IL-5 has been observed in serum from the immunized mice by Western blot analysis (Fig. 6). All of these results suggested that RAMPS also can modulate the quality of the immune responses and promote a balanced Th1/Th2 immune responses against FMD in mice.

The oral administration of 0.05 g RAMPS shows the strongest improvement on the immune response to FMDV but not the largest dose 0.1 g as indicated in Figs. 1–5. Many herbal extracts show a dose-dependent effect on the immune responses. For example, Sun et al. (2009) observed the highest serum OVA-specific antibody titers at a dose of 50 μg AEPS when the polysaccharides from the roots of *A. eriantha* (AEPS) (25, 50, 100 μg) administered subcutaneously with OVA (100 μg) in mice; Song et al. (2009) reported the highest IgG response in mice immunized with FMDV antigen plus 10 μg of GSLS (ginseng stem-leaf saponin) when mice were subcutaneously immunized with FMDV antigen with GSLS (1, 5, 10 and 20 μg). The exact mechanisms behind this phenomenon are unclear, but the reasons might be activation of suppressor T cell (Ts cells) at the high dose or presence of cytotoxic contaminants in the herbal extracts.

In conclusion, the fraction of polysaccharide in RAM may contribute to the oral adjuvant activity of RAM. After oral administration of RAMPS, a FMD vaccine induced significantly higher specific IgG and the IgG isotype responses in association with up-regulated serum IFN-γ and IL-5. Administration of RAMPS also significantly increased splenocyte proliferative responses to ConA, LPS and FMDV, as well as mRNA expression of Th1/Th2 cytokines (IFN-γ/IL-4) and transcription factors (T-bet/GATA-3) by splenocytes. Considering the oral adjuvant activities of RAMPS in FMD vaccination, its effect on the intestinal mucosal immunity should be investigated in our further study.

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