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Short communication. Enhancement of the immune responses to vaccination against foot-and-mouth disease in mice by oral administration of *Quillaja saponaria*-A and extracts of *Cochinchina momordica* seed

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Abstract

This study was designed to evaluate the effects of oral administration of extracts from *Cochinchina momordica* seed (ECMS) or *Quillaja saponaria*-A (Quil-A) on the immune responses in mice immunized with foot and mouth disease virus (FMDV)-serotype O vaccine. Forty-two imprinting control region (ICR) mice were randomly divided into seven groups of 6 animals in each group, and a dose of 400 µg of Quil-A or ECMS was orally administered for 1, 2 or 3 days. After that, the animals were subcutaneously immunized twice with FMD vaccine at 3-week intervals and blood samples were collected 2-weeks after boosting for measurement of FMDV-specific IgG and its subclasses. Spleens were collected for lymphocytes proliferation assay. Results indicated that serum FMDV-specific IgG and the IgG subclass responses were significantly enhanced in mice orally administered ECMS or Quil-A when compared with the control group ($p < 0.05$). Lymphocytes proliferation response to FMD vaccine was significantly enhanced by ECMS compared with the control ($p < 0.05$). This study illustrates that ECMS induced immunomodulatory effects and performed better than Quil-A.

Additional key words: adjuvant; ELISA; oral immunization; vaccine.

There is growing evidence that medicinal herbs and their ingredients enhance immune responses to vaccination against infectious diseases (Liu & Xiao, 1992; Rajput *et al.,* 2007a). Medicinal herbs have a variety of effects on immune responses and co-administration of vaccine with herbal extracts shows an increase in antibody response as well as enhancement in proliferative response of T cells (Hu *et al.,* 2003; Sun *et al.,* 2007). Some adjuvants such as *Quillaja saponaria*-A extracts (Quil-A) or extracts from *Cochinchina momordica* seed (ECMS) have proven to be very effective for enhancing immune responses with the commercial foot-and-mouth disease (FMD) vaccine (Xiao *et al.,* 2007a,b,c). Quil-A is produced from the bark of *Quillaja saponaria* Molina, a tree of the rose family which is indigenous to South America; it is a triterpenoid saponin which has been used over the past three decades as adjuvant in vaccines (Remington *et al.,* 1918; Kensil, 1996). ECMS is a crude extract made from seed of the Chinese traditional medicinal plant *Momordica cochinchinensis* and its immunomodulatory effects have been reported in vaccines against influenza-H5N1 (Rajput *et al.,* 2007b), infectious bursal disease (Rajput *et al.,* 2010) and newcastle disease (Xiao *et al*., 2009) in chicken. The immunomodulatory effect of ECMS was also reported against foot-and-mouth disease vaccination in Guinea pigs and pigs (Xiao *et al*., 2007c), and in mice (Sakwiwatkul *et al.,* 2010). Traditionally, the seed has been used for a variety of ailments including inflammatory swelling, diarrhea, and

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Abbreviations used: ECMS (extracts from *Cochinchina momordica* seed); FMD (foot-and-mouth disease); ICR (imprinting control region); MTT (methylthiazol tetrazolium assay); PBST (phosphate buffer saline + Tween-20); Quil-A (*Quillaja saponaria*-A); SI (stimulation index).

supportive skin infections in human and animals (Gao, 2005).

The present study was designed to investigate the effects of oral administration of Quil-A or ECMS on the immune responses in mice immunized with a commercial serotype-O FMDV vaccine.

The ECMS was extracted following Xiao *et al.* (2007a). Quil-A was supplied by Desert King Chile Ltd., Santiago, Chile. Commercially available FMD vaccine (serotype-O) adjuvanted with oil was produced by Lanzhou Veterinary Research Institute (LVRI), China.

Forty-two female imprinting control region (ICR) mice, purchased from Zhejiang Experimental Animal Center (Hangzhou, China), were housed in polypropylene cages with sawdust bedding in hygienically controlled environment and provided standard food and water *ad libitum*. Mice were randomly distributed into seven groups, each group comprising six animals. The mice of each group were treated as shown in Table 1. Briefly, each mouse were orally administered 400 µg (800 µg mL^{-1}) Quil-A or ECMS using oral syringes medicinal dropper to groups Quil-A1 or ECMS1 (on Day 3 and boosted on Day 25), Quil-A2 or ECMS2 (on Days 2 and 3 and boosted on Days 24 and 25) and Quil-A3 or ECMS3 (on Days 1, 2 and 3 and boosted on Days 23, 24 and 25). Control group was orally administered saline (0.9% NaCl) (on Days 1, 2 and 3 and boosted on Days 23, 24 and 25). Total volume of liquid orally administered to each mouse was 0.5 mL. On Day 4, all animals (including control) were subcutaneously injected with 200 µL of FMDV (serotype-O) vaccine and boosted with 3-weeks interval (on Day 26). The group receiving saline and FMD vaccine served as control. Two weeks after the booster dose (Day 40), blood samples were collected from all groups for measurement of serum FMDV-specific IgG and IgG subclasses. On the same day, the spleens from group Quil-A3, ECMS3 and control were collected for lymphocytes proliferation assay.

Serum samples were analyzed for measurement of FMDV-specific IgG antibodies and isotypes by an indirect double antibody sandwich enzyme-linked immunosorbent assay (ELISA) as described by Song *et al.* (2009). All the wells of polyvinyl 96-well microtitre plates were coated with 50 µL rabbit anti-FMDV (type 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. PBST was also used as a diluent and washing solution in all subsequent steps. Thereafter, 50 µL FMDV (type O) antigen (LVRI) (1:3 dilution) was added and incubated at 4°C for 2 h. After wash, 50 µL of serum samples (1:50) was added to duplicate wells and incubated at 37°C for 1 h. Following five washings, 50 µL of goat anti-mice IgG (1:500) (Kirkegaard, Perry Lab, MD, USA) was added to all wells and incubated at 37°C for 1 h. For subclass, 100 µL of biotin conjugated goat anti-mouse IgG1, IgG2a, IgG2b or IgG3 (1:600 dilution, Santa Cruz Biotechnol Inc, CA, USA) was added to the corresponding plate and then incubated for 1 h at 37°C. After washing, 100 µL of horseradish peroxidase conjugated anti-biotin (BD Biosciences, Pharmingen, USA) diluted 1:4000 in PBST was added to each well and incubated for 1 h at 37°C. Plates were washed again with PBST. Fifty microliters of TMB solution (100 µg mL^{-1} of 0.1 M citrate-phosphate, pH 5.0) was added to each well and incubated for 15 min

Table 1. Treatment schedule of *Quillaja saponaria*-A (Quil-A), extracts from *Cochinchina momordica* seed (ECMS) and foot and mouth disease (FMD) vaccine to mice (n = 6)

Groups	Immunization				Sample collection	
	Oral administration (400 µg Quil-A or ECMS)			Injection (200 µL FMD vaccine)	Blood	Lymphocytes
	Days 1 and 23	Days 2 and 24	Days 3 and 25	Days 4 and 26	Day 40	Day 40
Control (Saline)	–	–	–	+	+	+
Quil-A1	–	–	+	+	+	–
Quil-A2	–	+	+	+	+	–
Quil-A3	+	+	+	+	+	+
ECMS1	–	–	+	+	+	–
ECMS2	–	+	+	+	+	–
ECMS3	+	+	+	+	+	+

+: activity. –: inactivity.

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.

Mice were sacrificed and spleens were collected under aseptic conditions for lymphocyte proliferation assay as described previously (Li *et al.,* 2009). In brief, spleens were minced and passed through a fine steel mesh to obtain a homogeneous cell suspension. Cells were suspended in RPMI-1640 (Gibco, Bio-cult, Glasgow, UK) containing 300 mg glutamine L^{-1}, 2 g sodium bicarbonate L^{-1}, 10% calf serum and 50 µg gentamicin mL^{-1}. Cells were counted with a haemocytometer by trypan blue exclusion technique and suspension was adjusted to 3×10^6 viable cells mL^{-1}. The assay was carried out in a flat-bottom 96-well microplate, where the cells in 100 µL medium were cultured in a humid atmosphere in 5% CO_2 at 37°C for 68 h. Wells with FMDV (O type) at 50 µg mL^{-1} were used as stimulators while wells without mitogens served as control. After incubation, 50 µL of MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide] (Amresco, Cleveland, OH, USA) solution (2 mg mL^{-1}) was added to each well, and incubated for another 4 h. The plates were then centrifuged (400 g, 5 min) and the untransformed MTT was removed carefully using a pipette. To each well, 150 µL of a DMSO working solution (144 µL of DMSO with 6 µL 1 N HCl) was added and incubated for 15 min at 37°C to dissolve crystallisable formazan. The plates were read by an ELISA reader at 490 nm. The stimulation index (SI) was calculated based on the following formula: SI = OD value for mitogen-cultures/OD value for non-stimulated cultures. Data analysis was performed with SPSS software (vers. 11.5, SPSS Inc., Chicago, IL, USA) at 0.05 level of significance.

Responses of FMDV-specific IgG antibodies were significantly higher ($p < 0.05$) in mice orally administered Quil-A or ECMS as compared to the control group ($p < 0.05$) (Fig. 1a). A significant increase in IgG1 antibody level in serum was observed in mice that received ECMS for Day 1 or Day 3 as compared to the control group. Similarly, a significant increases of IgG2a and IgG3 levels were recorded in all groups of mice treated with Quil-A or ECMS ($p < 0.05$) as compared to the control (Fig. 1b). IgG2b antibody level was significantly higher than control in mice administered ECMS on Day 1. The proliferative response of Quil-A and ECMS on lymphocytes is shown in Fig. 1c. Stimulating index (SI) for the proliferation in response to FMDV was significantly increased ($p < 0.05$) in mice of group ECMS3 (which received ECMS three days before vaccination), when compared to the control.

In the present work, the enhancement of immune responses to vaccination against FMD was studied in mice model by oral administration of Quil-A or ECMS. Quil-A is a saponin fraction successfully used for veterinary applications (Dalsgaard, 1987). Significant in-

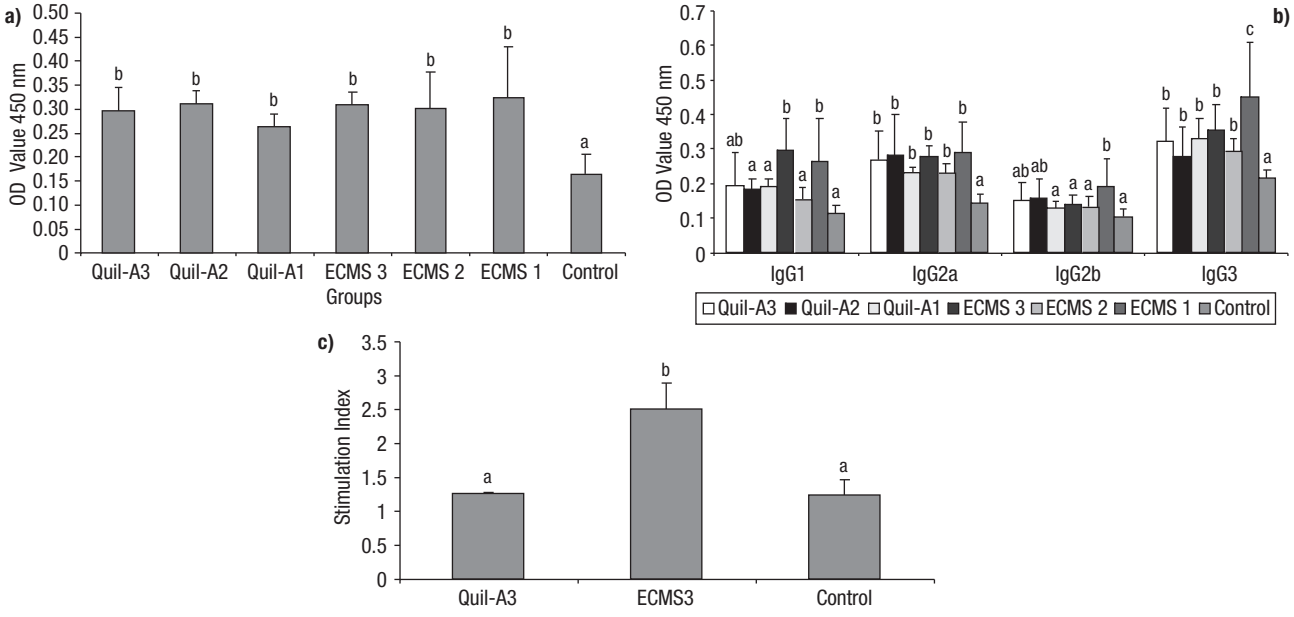

Figure 1. Immune response of mice to foot and mouth disease (FMD) vaccine (type O) with or without oral administration of Quil A or ECMS adjuvants. FMDV (type O) specific IgG antibodies (a), FMDV (type O) specific IgG subclasses (b) and lymphocytes proliferation (c). Different letters a,b,c indicate significant differences between groups ($p < 0.05$).

crease of humoral immune response has been reported in pigs immunized with FMD vaccine supplemented with Quil A (Xiao *et al.,* 2007b). ECMS has also been reported to have an adjuvant effect in FMD vaccination, with a much lower hemolytic activity (Xiao *et al.,* 2007a). However, to our understanding, this is the first report of the adjuvant activity of Quil-A or ECMS by oral administration. Oral administration of these two adjuvants, significantly increased serum specific IgG and its subclasses in mice inoculated with FMD vaccine.

Humoral immune response has been considered to be an important defense mechanism in FMD virus infection (Meloen *et al.,* 1979; Brown, 1995), and the contribution of antibodies to the major immune defense against the virus is clear (McCullough *et al.,* 1988, 1992). Studies with animal models (McCullough *et al.,* 1986, 1988) have shown that specific antibodies play an important role in the immune defense against FMD virus. However, poor humoral immune responses to FMD vaccination, in laboratory animals and pigs, have been reported previously (Shi *et al.,* 2007; Xiao *et al.,* 2007b). Fig. 1a shows that oral administration of Quil-A or ECMS significantly increased serum FMDV-specific IgG antibody to a commercial FMD vaccine. This finding corroborates with Sakwiwatkul *et al.* (2010).

Study of the IgG isotypes elicited in response to viral infection or immunization is therefore important when considering the mechanisms of *in vivo* protection (Pérez Filgueira *et al.,* 1995). The formation of IgG2a is typical for the Th1 response, while IgG1 is typical for Th2 response (Giese, 1998). Both IgG2a and IgG2b isotypes are the most effective in complement activation as well as in antibody-mediated cellular responses (Klaus *et al.,* 1979; Kipps *et al.,* 1985). Present study showed that the oral administration significantly increased the IgG subclasses (Quil-A, IgG2a, IgG3 and ECMS; IgG1, IgG2a, IgG2b and IgG3) as depicted in Fig. 1B. So, it is concluded that Quil-A induced Th1 type immune response while ECMS produced both Th1 and Th2 type responses. Th2 type cells have been reported to be efficient in inducing an antibody response to protein antigens (Rodriguez *et al.,* 1996).

Present study revealed that IgG3 was the predominant isotype among total anti-FMDV IgG subclasses (Fig. 1b). The IgG3 subclass presents the tendency to form aggregates by non-covalent bonds between the Fc region of other adjacent IgG3 molecules and fixes complement poorly (Grey *et al.,* 1971; Greenspan & Cooper, 1992). It has also been reported that all monoclonal antibodies (MAbs) that caused FMDV aggregation were of the IgG3 subclass (Baxt *et al.,* 1984). Viral neutralization produced by the mechanism of aggregation might be isotype-related (Baxt *et al.,* 1984; Greenspan & Cooper, 1992).

Lymphocytes of the three groups (Quil-A, ECMS3 and control) were stimulated by FMDV (type O) antigen (Fig. 1c). FMDV (type O)-induced lymphocyte proliferation response was significantly enhanced in ECMS 3 group, but there was no change in Quil-A group. This observation suggests that ECMS has the potential to activate T as well as B cells. In present study, it is concluded that ECMS induced more immunomodulatory effects compared to Quil-A and more safe as reported previously.

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