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ORIGINAL ARTICLE

E a a *Cochinchina momordica* a a -a a a (O a A a 1)

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ABSTRACT

The extract from ECMS was investigated for its effect on the humoral immune responses to foot-and-mouth disease vaccination. Fifty-six mice were randomly divided into seven groups with eight animals in each. Mice in groups 5 to 7 were subcutaneously (s.c.) injected with 0.5 mg DEX daily for 4 days to induce immunosuppression. The animals were then orally given ECMS (200 μg in 250 μl saline) in groups 3 and 6 or 250 μl saline in group 2, or s.c. injected with ECMS (50 μg in 100 μl saline) in groups 4 and 7 or 100 μl saline in group 5. After that, the animals in groups 2 to 7 were s.c. immunized twice with 100 μl of commercial oil-adjuvanted bivalent FMDV vaccine (serotypes O and Asia 1) at intervals of 21 days. Mice in group 1 received injection of 100 μl saline only. After 2 weeks, blood was sampled to determine FMDV-specific IgG and isotype IgG1, IgG2a, IgG2b and IgG3. Results indicated that oral administration or s.c. injection of ECMS augmented responses of specific IgG and most IgG isotypes. Giving ECMS tended to enhance serum-specific IgG and IgG isotype responses of mice immunosuppressed by s.c. injection of DEX. Considering the safety and immunomodulatory effect of ECMS in both normal and immunosuppressed mice demonstrated in the present study, this extract deserves further investigation to evaluate its potential in improving FMD vaccination in farm animals such as pigs, sheep and cattle.

Key words *Cochinchina momordica* eed , foo -and-mo h di ea e, accine.

Foot-and-mouth disease is caused by FMDV, which is the prototype member of the *Aphthovirus* genus, *Picornaviridae* (1) and occurs as seven major serotypes: A, O, C, Asia 1, SAT 1, SAT 2 and SAT 3, but a large number of subtypes are found within each serotype (2, 3). The disease mainly affects the cloven-hoofed animals. FMD has been recorded as one of the most important animal diseases in the world by the World Organization for Animal Health (OIE) due to the rapid transmission of FMDV among susceptible animals (4). Vaccination is a common practice against FMD in many countries. However, failure to elicit effective immune responses by vaccination has been frequently reported (5–7). In agreement with this, Hao *et al.* (8) observed that only 31.9% out of 91 pigs vaccinated against FMD (type O) produced serum antibody titers needed for immune protection. Hence, currently available vaccines should be improved in order to effectively prevent infectious diseases in animals. Higher dosage of vaccine

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List of Abbreviations: DEX, de ame ha one; ECMS, *Cochinchina momordica* eed ; FMD, foo -and-mo h di ea e; FMDV, FMD ; Ig, imm noglob lin; OVA, o alb min.

or higher frequency of vaccination and use of adjuvants such as saponins and a variety of immunomodulators are some approaches to improve the immune response to vaccination (9–14).

Semen momordicae is the seed of *Momordica cochinchinensis* (Lour.) Spreng. mainly growing in Southeast Asian countries and southern China (15). Traditionally, the seeds are used to treat inflammatory swelling, diarrhea and suppurative skin infections in human beings and animals (16). From the seeds, Iwamoto *et al*. (17) have isolated momordica saponins I and II. According to the chemical analysis, momordica saponin I is a triterpenoid saponin containing disaccharide chains, and momordica saponin II is structurally similar to quillaic acid. Our previous study has shown that the addition of (ECMS extract in a commercial foot-and-mouth disease vaccine can significantly enhance immune responses in pigs (18). ECMS given s.c. has also been shown to exert an adjuvant effect on the immune response particularly at a humoral level to OVA (19) in mice and to influenza vaccination (H5N1) in chickens (20). In the present study, the immunomodulatory effects of ECMS by the oral or the s.c. route on the humoral immune response of normal and immunosuppressive mice against vaccination of bivalent FMDV serotypes O and Asia I vaccine were evaluated. To induce immunosuppression in ICR mice, DEX was injected s.c. prior to giving ECMS and bivalent FMDV vaccine, as DEX has been shown to inhibit several aspects of cell-mediated immunity, including antigen- and mitogen-induced lymphocyte proliferation (21) and is commonly used to produce an immunosuppressive model in mice.

MATERIALS AND METHODS

E ia a a

Female ICR mice with a bodyweight of 19–22 g were purchased from Shanghai Laboratory Animal Center Co. Ltd (Shanghai, China) and kept in cages bedded with sawdust in a controlled environment. Feed and water were supplied *ad libitum*. The study was conducted in accordance with the Guidelines for Keeping Experimental Animals released by the Ministry of Health of China.

E a ***Cochinchina momordica***

ECMS was prepared following the method described by Xiao *et al.* (18). The powder of *Cochinchina momordica* seeds was submerged in 50% ethanol for 24 hr. The mixture was refluxed in a round bottom flask three times at 90°C, with 2 hr for each reflux, and the ethanol was then evaporated with a R502B rotary evaporator (Shenko Tech Co. Ltd, Shanghai, China). After that, the extract was washed with diethyl ether to eliminate the substance soluble in ether. The saponin fraction was dissolved in water saturated *n*-butanol and the butanol-soluble portion was passed through a chromatography column with macroporous resin D101A (Hai Guang Chemical Co. Ltd, Tianjin, China) to remove impurities. Refined ECMS was harvested by removing the liquid eluted from the column.

FMDV a a a a

FMDV vaccine was a commercial oil-adjuvanted bivalent vaccine (serotypes O and Asia I) made by Xinjiang Tiankang Animal Science Bio-Technology Co., Yinin, China. Dexamethasone sodium phosphate (DEX) (5 mg/ml) was a product of Sishui Xierkang Pharmaceutical Co., Shishui, China.

A a DEX, ECMS a FMDV a

Fifty-six ICR mice were randomly allocated into seven groups, and each group consisted of eight animals. The mice were treated according to the schedule described in Table 1. Briefly, mice in groups 5 to 7 were s.c. injected with 0.5 mg dexamethasone daily for 4 days to induce immunosuppression. The animals were then orally given

Table 1. Trea men ched le of DEX, ECMS and FMDV accine o ICR mice (n = 8/gro p)

Gro p no.	Gro p	Trea men Da of admini ra ion	DEX Da 1 4	ECMS Da 5 and 26	FMDV accine Da 6 and 27
1	Saline		/[a]	–[b]	–
2	Con rol		/	–	+
3	ECMS (oral)		/	+ (oral)	+
4	ECMS (.c.)		/	+ (.c.)	+
5	DEX		+	–	+
6	DEX + ECMS (oral)		+	+ (oral)	+
7	DEX + ECMS (.c.)		+	+ (.c.)	+

[a]Mice recei ed no rea men .
[b]Mice ere gi en aline onl .

ECMS (200 μg in 250 μl saline) in groups 3 and 6 or 250 μl saline in group 2, or s.c. injected with ECMS (50 μg in 100 μl saline) in groups 4 and 7 or 100 μl saline in group 5. After that, except the animals in group 1, all other animals were s.c. immunized with 100 μl oil-adjuvanted bivalent FMDV vaccine (serotypes O and Asia I). Mice in group 1 received injection of 100 μl saline only. Three weeks after the first immunization, a boosting immunization, and administration of DEX and ECMS before boosting were carried out in the same manner as described above.

Sa a a a b a

Blood samples were collected 2 weeks after boosting immunization for the detection of FMDV-specific IgG responses and IgG isotypes. The bodyweight of each mouse was measured on days 0, 7, 11, 32 and 46 to evaluate the effects of DEX and ECMS on the mean bodyweight of mice.

M a FMDV (O a A a I)- I G a I G

An indirect double antibody sandwich ELISA was used for the determination of serum IgG and the isotypes as previously reported by Xiao *et al.* (18). In brief, the wells of polyvinyl 96-well microtiter plates were added to 50 μl rabbit anti-FMDV (type O or Asia I) antibody (Lanzhou Veterinary Research Institute, Lanzhou, China) in 0.05 M carbonate/bicarbonate buffer, pH 9.6 (1:1000) and incubated overnight at 4°C. After washing the wells with PBS containing 0.05% Tween-20 (PBST), they were incubated with 3% skimmed milk for blockage at 37°C for 2 hr. In the later steps, PBST was used as a diluent as well as a washing solution. Afterward, the wells were added to 50 μl FMDV antigen (LVRI) (for detection of serotype O-specific IgG, antigen was diluted 1:3 with 3% skimmed milk/PBS and for measurement of serotype Asia I-specific antibody responses, antigen was diluted 1:10 with 3% skimmed milk/PBS) and incubated at 4°C for 2 hr. Following washing, the wells were added in duplicate with 50 μl of serum samples (1:50) and kept warm at 37°C for 1 hr. After another washing, all wells were added to 50 μl goat antimouse IgG (1:1000) (Kirkegaard, Perry Lab., Gaithersburg, MD, USA) and incubated at 37°C for 1 hr. For the determination of subclasses, 100 μl biotin-conjugated goat antimouse IgG1 or IgG2a or IgG2b or IgG3 (1:600 dilution, Santa Cruz Biotechnology Inc., Santa Cruz, CA) was added to the corresponding plate and then incubated for 1 hr at 37°C. Subsequent to washing, each well was added to 100 μl horseradish-peroxidase-conjugated anti-biotin (BD Biosciences Pharmingen, Franklin Lakes, NJ, USA) (1:4000 in PBST) and incubated for 1 hr at 37°C. One more wash was carried out and each well was added to 50 μl TMB solution (100 μg/ml 0.1 M citrate–phosphate, pH 5.0). Following development for 15 min at 37°C, 50 μl of 2 M H_2SO_4 was added to each well to stop the reaction. An automatic ELISA plate reader was used to read the optical density of the plate at 450 nm.

S a a a a a a

All data were subjected to one-way analysis of variance. Bonferroni method of multiple comparisons was carried out to compare the parameters among groups (22). For all the tests, $P < 0.05$ was considered significant. Data were expressed as mean ± SD.

RESULTS

E ECMS a b

The effects of ECMS on the bodyweight of mice are shown in Table 2. The average bodyweights of mice in dexamethasone-treated groups were significantly lower than untreated groups on day 11. Otherwise, no statistical difference was observed among groups.

Table 2. Effec of DEX and ECMS on he mean bod eigh (g; mean ± SD) of mice ($n = 8$/gro p)

Gro p no.	Gro p	Da 0	Da 7	Da 11	Da 32	Da 46
1	Saline	20.53 ± 0.52	25.05 ± 1.41	24.49 ± 2.41[a]	32.01 ± 2.31	34.14 ± 3.27
2	Con rol	20.45 ± 1.42	23.96 ± 1.04	25.63 ± 1.43[a]	30.79 ± 2.35	32.23 ± 3.34
3	ECMS (oral)	20.45 ± 1.14	22.76 ± 1.28	25.34 ± 1.87[a]	31.31 ± 2.80	33.28 ± 2.75
4	ECMS (.c.)	20.46 ± 0.73	25.53 ± 1.42	27.49 ± 1.38[a]	31.93 ± 1.80	31.99 ± 4.08
5	DEX	20.35 ± 0.72	24.26 ± 0.81	22.79 ± 1.07[b]	31.34 ± 2.35	33.09 ± 2.26
6	DEX + ECMS (oral)	20.46 ± 1.09	25.20 ± 1.15	23.93 ± 1.11[b]	31.54 ± 2.00	33.48 ± 2.20
7	DEX + ECMS (.c.)	20.35 ± 0.92	24.09 ± 1.86	22.85 ± 1.81[b]	29.88 ± 2.80	32.38 ± 2.95

Mean bod eigh i h differen per crip le er are igni can l differen .

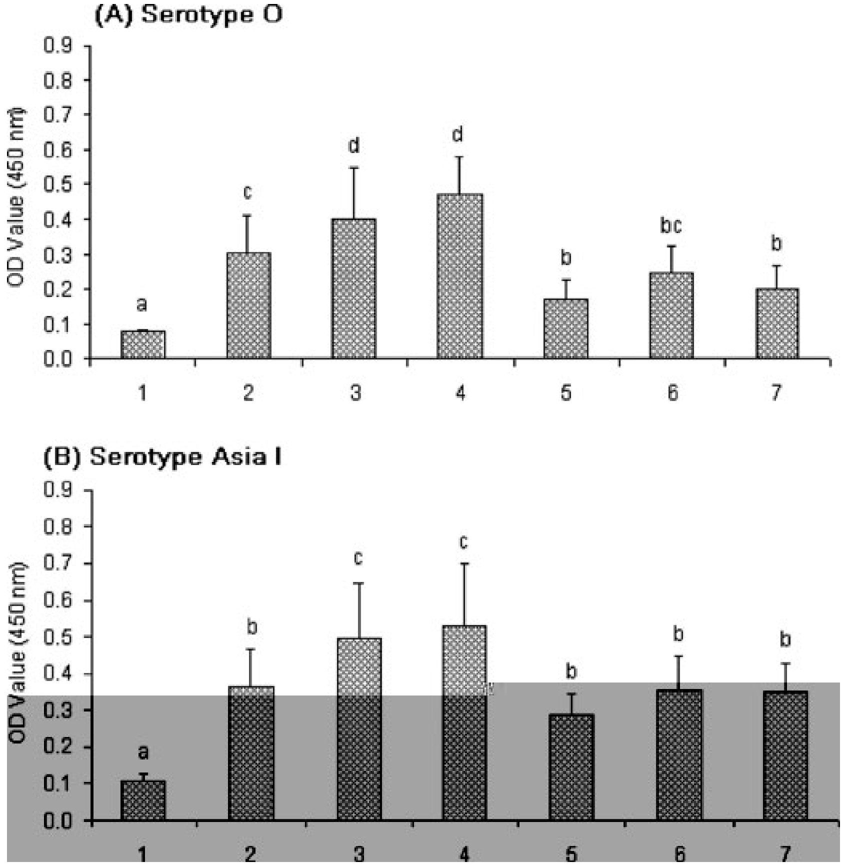

Fig. 1. Ser m IgG re pon e o FMDV ero pe O (A) and A ia I (B). Mice (n = 8/gro p) ere rea ed i h (1) aline ol ion; (2) FMDV an igen; (3) oral ECMS + FMDV; (4) injec ion ECMS + FMDV; (5) DEX + FMDV; (6) DEX + oral ECMS + FMDV and (7) DEX + injec ion ECMS + FMDV. Af er boo er imm ni a ion, blood ample ere collec ed for he mea remen of FMDV- peci c IgG re pon e b an indirec ELISA. Val e are pre en ed a mean ± SD. Bar i h differen le er deno e a i icall igni can difference (P < 0.05).

FMDV (O a A a I)- I G

The effects of ECMS on the humoral immune responses against FMDV serotype O and Asia I are depicted in Figure 1. Both oral administration of ECMS (200 μg) and s.c. injection of ECMS (50 μg) significantly ($P < 0.05$) increased serum IgG responses to FMDV serotypes O and Asia I when compared with the control (group 2 in Fig. 1). Injection of DEX suppressed IgG responses to FMDV serotype O significantly or to FMDV serotype Asia I numerically when compared with the control (group 2 in Fig. 1) but oral administration or injection of ECMS (groups 6 and 7) numerically increased IgG levels when compared with the control (group 5 in Fig. 1).

FMDV (O a A a I)- I G

The effects of ECMS on IgG isotypes IgG1, IgG2a, IgG2b and IgG3 to FMDV serotypes O and Asia I are shown in Figure 2. Oral administration and injection of ECMS tended to enhance all IgG isotype responses to immunization of bivalent FMDV vaccine (serotypes O and Asia I) when compared with the control in which only FMDV vaccine was injected. Injection of DEX suppressed IgG isotype responses but oral administration or injection of ECMS tended to elevate IgG isotype levels.

DISCUSSION

Enhanced humoral immune response of mice to vaccination of bivalent FMDV serotypes O and Asia I vaccine has been demonstrated by oral administration or s.c. injection of an extract made from ECMS. After oral administration of ECMS (200 μg) or s.c. injection of ECMS (50 μg), immunization of a commercial FMDV vaccine induced significantly higher serum specific IgG and most IgG isotype responses than in mice given saline solution alone. In addition, giving ECMS tended to enhance serum-specific IgG and IgG isotype responses of mice immunosuppressed by s.c. injection of dexamethasone.

The mouse model has been used to study the immunity of a host against FMDV infections (23–25). Salguero *et al.* (23) have reported that mice immunized with conventional inactivated FMDV vaccine can be protected against challenge with a lethal dose of FMDV. Wong *et al.* (25) have

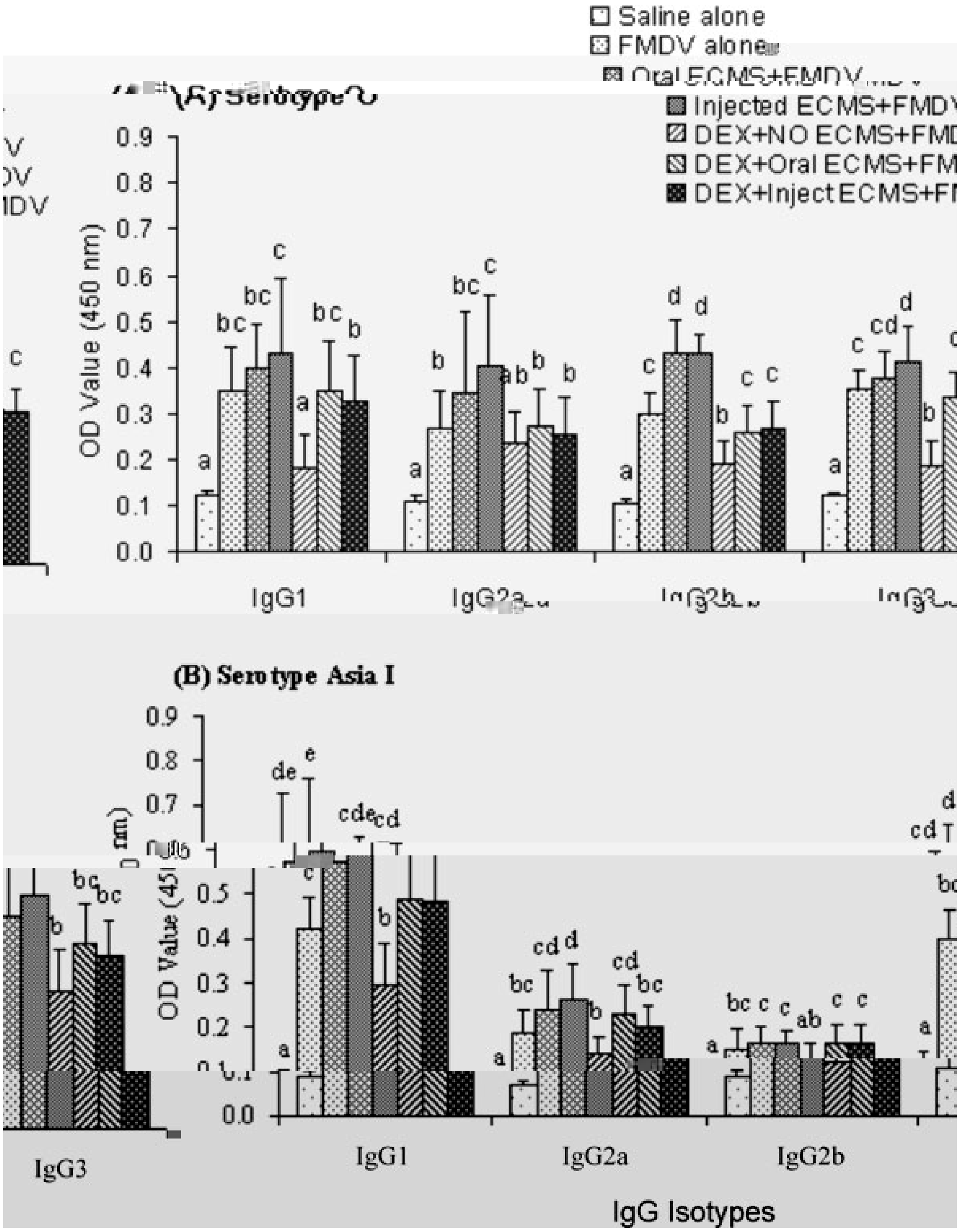

Fig. 2. Ser m IgG i o pe re pon e o FMDV ero pe O (A) and A ia I (B). Mice (n = 8/gro p) ere rea ed i h (1) aline ol ion; (2) FMDV an igen; (3) oral ECMS + FMDV; (4) Injec ion ECMS + FMDV; (5) DEX + FMDV; (6) DEX + oral ECMS + FMDV and (7) DEX + injec ion ECMS + FMDV. Af er boo er imm ni a ion, blood ample ere collec ed for he mea remen of FMDV- peci c IgG i o pe IgG1, IgG2a, IgG2b and IgG3 re pon e b an indirec ELISA. Val e are pre en ed a mean ± SD. Bar i h differen le er deno e a i icall igni can difference ($P < 0.05$).

observed that a FMD DNA vaccine inducing an immune response in mice can also elicit protection in swine against FMD infection. Humoral immune response has been reported as an important defense mechanism against FMD virus (26, 27), and the contribution of antibodies to the major immune defense against the virus is clear (28, 29). Studies with animal models (28, 30) have suggested that a specific humoral response is vital in the immunity against FMD virus. However, poor antibody response to FMD vaccination has been previously reported in both experimental animals and pigs (24, 31). Figure 1 shows that oral administration or s.c. injection of ECMS significantly enhanced serum FMDV serotypes O and Asia I-specific IgG levels to a commercial bivalent FMDV vaccine. This result is consistent with our previous studies where immune responses were enhanced by co-administration of ECMS with commercial FMDV vaccine in pigs (18) or avian influenza (H5N1) vaccine in chickens (19).

Immunosuppression constitutes one of the reasons for poor immune responses to vaccination. In the present study, daily s.c. injection of DEX for 4 days effectively inhibited the immunity of mice, resulting in suppressed IgG and IgG isotype responses to FMDV vaccination (groups 5–7 on day 11 in Fig. 2). Nevertheless, oral administration or s.c. injection of ECMS caused numerically higher serum-specific IgG and IgG isotypes than in mice without ECMS treatment, indicating ECMS might have an immunomodulatory effect on immunosuppressed mice.

A conventional approach for the improvement of the efficacy of vaccination is to add adjuvant to vaccines. Adjuvant used for this purpose should be safe enough to induce minimal adverse effects to prove acceptable for use in healthy individuals. Many natural products have been reported having immunomodulatory properties, whereas their modes of action are usually unclear. Purification of the herbal extracts is usually difficult, and irritation will take place when unpurified herbal extracts are co-injected with immunizing antigens. As traditional medicinal herbs are generally given by oral route, oral use of immunomodulators can avoid the side-effects found in parenteral administration. For example, oral administration of crude saponins made from the bark of the *Quillaja* tree has been proven to have immunopotentiating activity with moderated toxicity, although they are toxic when given parenterally (32, 33). In the present study, neither abnormal behavior nor adverse side-effects were found in mice throughout the experiment, and there was no significant difference in the bodyweight between the mice given ECMS and the control mice given saline solution as indicated in Table 2 (groups 3 and 4 to group 2, groups 6 and 7 to group 5, respectively), suggesting that oral administration of ECMS is safe.

Immunosuppression constitutes one of the reasons for poor immune responses to vaccination. In this study, daily s.c. injection of DEX for 4 days effectively inhibited the immunity of mice, resulting in suppressed IgG and IgG isotype responses to FMDV vaccination (groups 5–7 on day 11 in Fig. 2). However, oral administration or s.c. injection of ECMS caused numerically higher serum-specific IgG and IgG isotypes than in mice without ECMS treatment, indicating that ECMS might have an immunomodulatory effect on immunosuppressed mice.

In summary, oral administration or s.c. injection of ECMS augmented responses of serum-specific IgG and most IgG isotypes to immunization of a commercial FMDV (serotypes O and Asia 1) vaccine in mice. Giving ECMS tended to enhance serum IgG and IgG isotypes of mice immunosuppressed by s.c. injection of DEX. Considering the safety and immunomodulatory effect of ECMS in both normal and immunosuppressed mice demonstrated in the present study, this extract deserves further investigation to elucidate its potential in improving FMD vaccination in farm animals such as pigs, sheep and cattle.

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