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Adjuvant effect of an extract from *Cochinchina momordica* seeds on the immune responses to ovalbumin in mice

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Abstract The seed of the *Momordica cochinchinensis* (Lour.) Spreng as a traditional Chinese medicine has been utilized in China for more than 1 200 years. It is traditionally used for the treatment of inflammatory swelling, scrofula, tinea, diarrhea as well as suppurative skin infections such as sore, carbuncles, furuncles and boils in both humans and animals. In this study, an extract from *Cochinchina momordica* seeds (ECMS) is evaluated for its adjuvant effect and safety. The results suggest that when co-administered with ECMS in Balb/c mice, ovalbumin (OVA, 10 µg) may induce significantly higher specific antibody production than OVA used alone ($P < 0.05$). Analysis of antibody isotypes indicates that the ECMS can promote the production of both IgG₁ and IgG_{2a}, but favor the IgG_{2a}. Splenocyte proliferative responses to concanavalin A, lipopolysaccharides or OVA are significantly higher in mice immunized with OVA mixed with ECMS than immunized with OVA alone or mixed with aluminum hydroxide ($P < 0.05$). No local reactions and negative effects on the body weight gain occurred after the injection of OVA mixed with various amounts of ECMS in mice. Therefore, the ECMS is safe for injection and can be used as a potential vaccine adjuvant biasing the production of IgG_{2a} in mice.

Keywords extract of *Cochinchina momordica* seed, adjuvant, *Momordica cochinchinensis* (Lour.) Spreng

1 Introduction

There has been an extensive research on the development of new vaccine adjuvants, but aluminum salts remain the only adjuvant registered for human beings and most veterinary vaccines, primarily because aluminum salts prove to be safe (Rivera et al., 2003). Unfortunately, vaccines adjuvanted with aluminum have a number of limitations (Tizard, 1996),

such as the short duration of the antibody response, the poor cell mediated immunity and the formation of IgE antibodies, which may result in hypersensitive reactions. Recent researches suggest that the aluminum vaccine may be involved in a muscle ailment in human beings (Malakoff, 2000). Therefore, searching for an ideal vaccine adjuvant is still a challenge.

This study mainly focuses on the evaluation of a traditional Chinese medicine *Cochinchina momordica* seeds for its adjuvant properties. Seeds are from the plant *Momordica cochinchinensis* (Lour.) Spreng, which is a perennial vine and grows mainly in Southeast Asian countries such as Vietnam, Laos, Cambodia, Burma, the Philippines, Malaysia, Bangladesh and Thailand, and many provinces in southern China such as the provinces of Guangxi, Sichuan, Hubei, Hunan, Guizhou, Yunnan, Guangdong, Anhui, etc. (Xu, 1996). The local people, in the winter, collect fruits, and separate them from seeds which are then dried for medicinal purposes such as the treatment of inflammatory swelling, scrofula, tinea, diarrhea as well as suppurative skin infections such as sore, carbuncles, furuncles and boils in human beings and animals (Gao, 2005; Zheng et al., 1992). An ancient Chinese medical literature *Kai Bao Materia Medica* in the Song Dynasty (793 AD) described that the seeds had a therapeutic effect on the acute mastitis and anusitis in humans (Gao, 2005). The drug is now included in both Chinese Pharmacopeia (Chinese Pharmaceutical Codex Evaluation Committee, 2005) and Chinese Veterinary Pharmacopeia (Chinese Veterinary Pharmaceutical Codex Evaluation Committee, 2000). Chemical analysis shows that the *C. mormodica* seeds contain fatty acid, saponin, protein, α -spinasterol, oleanolic acid, momordica acid, etc. (Xu, 1996; Gao, 2005; Shang et al., 2002; Shang et al., 2000; Ding et al., 2005). Recent investigations prove that a crude liquid extract from the seeds has anti-tumor functions (Tien et al., 2005; Tsoi et al., 2005; Wong et al., 2004). Iwamoto et al. (1985) isolated two kinds of saponins called momordica saponins I and II from the seeds. According to his observation, momordica saponin I is gypsoside, which is the tri-terpenoid saponin containing disaccharide chains, and momordica saponin II is mostly the quillaic acid. Some

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saponins with tri-terpenoid structures exhibit vaccine adjuvant effects, such as the saponins extracted from the bark of *Quillaja saponaria* Molina (Dalsgaard, 1978) or those from the root of *Panax ginseng* C.A. Meyer (Rivera et al., 2003; Hu et al., 2003; Riveria et al., 2003). But no report has been found up to now regarding to its immunological effects on both humans and animals. However, based on the information from the above mentioned, it can be assumed that the seeds may have the properties of an adjuvant that augments the immune response to vaccine antigens. To prove this hypothesis, an extract from *C. momordica* seeds (ECMS) was prepared, and then its adjuvant effect on the immune response in mice immunized with ovalbumin (OVA) in combination with ECMS was evaluated. Since aluminum hydroxide gel (Al-gel) is a common adjuvant in most commercially available vaccines, this adjuvant was used as a reference in the experiment.

2 Materials and methods

2.1 Preparation of ECMS

Thirty kilograms of dried *C. momordica* seeds were purchased from a market of traditional Chinese herbs in Anhui Province, China, and the seeds were appraised by Zhejiang Institute of Veterinary Drug Control. The drugs were divided into three batches with each containing 10 kg of seeds. Seeds were crushed or ground into powder, which was then dissolved in 50% ethanol for 24 hours. The powder and ethanol mixture was put into a round-bottom flask. The mixture was refluxed three times at 90°C with each reflux taking two hours. The remaining ethanol was removed by a R502B rotary evaporator (Shenko Tech Co Ltd, Shanghai, China). The extract was then dissolved in water and washed with diethyl ether to remove the ethersoluble substance. After that, the saponin fraction was extracted with water-saturated n-butanol. The butanol-soluble fraction was purified by passing through a chromatography column with resin D101A (Hai Guang Chemical Co Ltd, Tianjin, China). A purified ECMS was obtained by evaporating the liquid eluted from the column.

2.2 Haemolysis of ECMS

Haemolytic activity of ECMS was assayed by incubating with red blood cells (RBC) from swine, sheep and cattle. In short, blood was washed three times with saline solution at 1 000 r/min for 10 min. Thereafter, the RBC was suspended in saline solution at a concentration of 0.5%. The RBC suspension was incubated with equal volumes of saline solution containing ECMS or Quil A (NOR-VET ApS, Denmark) at concentrations ranging from 1 to 500 µg/mL at 37°C for one hour. After that, samples were centrifuged and the optical density (OD) value of the supernatant was measured at 405 nm.

2.3 Adjuvant effect of ECMS

2.3.1 Preparation of ECMS solution

ECMS was dissolved in distilled water to make a solution containing ECMS of 1 mg/mL, and it was then filtered through a filter membrane with 0.2 µm pores. The solution was analysed for endotoxin by a gel-clot *Limulus* amoebocyte lysate assay (bath No. 050260, Zhanjiang A & C Biological Ltd, Zhanjiang, China). The endotoxin level in the ECMS solution was less than 0.5 endotoxin unit per mL.

2.3.2 Immunisation and sample collection

Thirty 5-week-old female mice were purchased from the National Experimental Rodents Center, Shanghai, China, and provided with a specific pathogen-free condition. The mice were randomly divided into 5 groups with 6 mice in each group. Each mouse in all the test groups was subcutaneously injected with a 0.89% saline containing 10 µg of ovalbumin (OVA, bath No. 0200902, Bio-tech Co. Ltd, Shanghai, China) and 0, 10, 50, 100 µg of ECMS or 50 µg of aluminum hydroxide gel (Al-gel, bath No. 20050726, Biological Medicine Factory, Animal Husbandry Industry Co Ltd, Jiangxi, China). Booster immunization was conducted three weeks after the first immunization. The details are shown in Table 1. Two weeks after the booster, blood samples were collected from the retro-orbital plexus to separate the serum, which was stored at -20°C until use, and the spleen cells were isolated for proliferation assay.

Table 1 Immunization protocol of mice with various OVA-ECMS formulations

| Group | No. of mice | OVA /µg | Adjuvant /µg |
|-------|-------------|---------|--------------|
| 1 | 6 | 10 | – |
| 2 | 6 | 10 | 10 ECMS |
| 3 | 6 | 10 | 50 ECMS |
| 4 | 6 | 10 | 100 ECMS |
| 5 | 6 | 10 | 50 Al-gel |

Note: Six Balb/C mice were immunized subcutaneously (200 µL) at a 3-week interval

2.3.3 Measurement of OVA-specific IgG

An indirect enzyme-linked immunosorbent assay (ELISA) was conducted to analyse the titres of anti-OVA antibodies in serum mainly as described by Hu et al. (2003). Briefly, after coating flat-bottomed 96-well plates (Gong Dong Medical Plastic Factory, Zhejiang, China) with OVA in 0.05 mol carbonate buffer, pH 9.6 (5 µg/mL), the plate was washed with 0.01 mol phosphate buffer solution (PBS) containing 0.05% of Tween 20 (PBS-T) (pH 7.4) and obturated to culture in (PBS) containing 1% of calf serum (pH 7.4) for one hour at 37°C. After that, twofold serial dilution of the serum was made in PBS with 0.5% calf serum (PBS-C) and the plates were cultured for two hours at 37°C. Subsequently,

horseradish peroxidase conjugated goat anti-mouse IgG (Chemicon International, Inc, Temecula California USA) in PBS with 0.5% of calf serum (1:500, v/v) was added to the plates (100 μ L per well), and cultured for two hours at 37°C. After being rewashed, the plates were cultured for 15 min at 37°C with TMB (Sigma Chemical, St. Louis, MO, USA) substrate (100 μ L per well). The reaction was stopped by adding 50 μ L of 2 mol H_2SO_4 to each well. The OD was read on an automatic ELISA plate reader (Dialab, GMBH, Austria) at 450 nm. The cut-off OD value was set as described by Frey et al. (Du, 1994). The antibody titre was defined as the highest dilution that gave a reading above the cut-off value.

2.3.4 Measurement of OVA-specific IgG₁ and IgG_{2a}

A flat-bottomed 96-well plate was coated as described above. After the coating, 100 μ L of diluted serum (1:800) was added to each well of the plate and cultured for one hour at 37°C. After the plate was washed, 100 μ L of biotin conjugated goat anti-mouse IgG₁ or IgG_{2a} diluted 1:600, (Santa Cruz Biotechnology Inc, California, USA) was added to each well of the plate, which was then cultured for one hour at 37°C. Then, the plate was rewashed and 100 μ L of horseradish peroxidase conjugated anti-biotin (BD Biosciences, Pharmingen, USA) diluted at 1:4 000 in PBS-C was added to each well to have another incubation for one hour at 37°C. After another washing cycle with PBS-T, the plates were incubated for 15 min at 37°C with TMB substrate (100 μ L per well). The reaction ended by adding 50 μ L of 2 mol H_2SO_4 to each well. The OD of the plate was read on an automatic ELISA plate reader at 450 nm.

2.3.5 Splenocyte proliferation assay

Spleen cells were harvested with aseptic procedures from the mice treated as mentioned above. The cells were suspended in RPMI-1640 (Gibco, Bio-cult, Glasgow, UK) containing 300 mg glutamine/L, 2 g sodium bicarbonate/L, 10% calf serum and 50 μ g getamycin/mL. Cell suspension was adjusted to 5×10^6 viable cells/mL by using the Trypan Blue exclusion test (Frey et al., 1998). Splenocyte proliferation was assayed as described previously (Concha et al., 1996). Briefly, the assays were carried out in a flat-bottom 96-well microplate. Wells containing 5×10^5 cells in 100 μ L medium were cultured in 5% CO_2 at 37°C. Concanavalin A (Con A, Sigma Chemical, St. Louis, MO, USA) at 5 μ g/mL or lipopolysaccharide (LPS, Sigma Chemical, St. Louis, MO, USA) at 5 μ g/mL or OVA at 25 μ g/mL in the well was used as stimulators. Wells without mitogens were used as controls. All the tests were carried out in triplicate. After incubation with Con A or LPS for 44 h, incubation with OVA for 92 h, 50 μ L of MTT (Amresco, Cleveland Ohio, USA) solution (2 mg/mL) 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 by a pipette. To each

well, 150 μ L of a DMSO working solution (144 μ L of DMSO with 6 μ L 1 mol HCl) was added and incubated for 15 min at 37°C to dissolve crystallisable formazan. The plates were read by an ELISA reader at 570 nm. The stimulation index (SI) was calculated based on the following formula:

$$SI = \text{OD value for mitogen-cultures} / \text{OD value for non-stimulated cultures.}$$

2.4 Statistical analysis

Comparisons between mouse groups with respect to the levels of serum antibody and stimulation index were made using Duncan's multiple range test. Probabilities less than 0.05 were considered statistically significant.

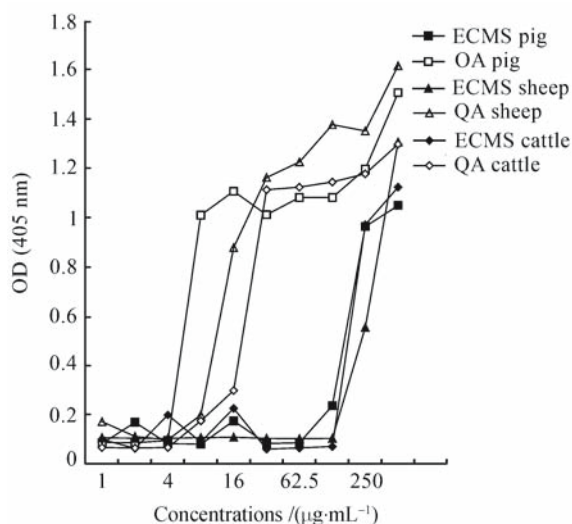
3 Results

3.1 Production of ECMS from *C. momordica* seeds

The amount of ECMS extracted from 3 extractions of *C. momordica* seeds at 10 kg at a time was 20.58 g, 22.10 g and 19.63 g, respectively. The average recovery rate of ECMS from the seeds was 2.08 g/kg. The extract was a yellowish powder with a special fragrance.

3.2 Hemolytic properties

The minimum hemolytic concentration of ECMS on RBC of swine, sheep and cattle was around 125 μ g/mL while Quil A was at 4 μ g, 8 μ g and 16 μ g/mL, respectively (Fig. 1).



The RBC suspension was incubated with saline solution containing ECMS or Quil A (QA) at concentrations ranging from 1 μ g to 500 μ g/mL at 37°C for 1 hour. After that, samples were centrifuged and the OD value of the supernatant was measured at 405 nm.

Fig. 1 Hemolytic activity of ECMS on RBC from swine, sheep and cattle

3.3 Local reaction at the injection site and effects of ECMS on the body weight gain of mice

No local reaction was found in all mice after injection. Table 2 shows that subcutaneous injection of OVA (10 μ g) mixed with various amounts of ECMS or 50 μ g of Al-gel in mice did not significantly influence the body weight gain as compared with injection of OVA only.

Table 2 Effects of subcutaneous injection of 10 μ L of OVA mixed with 0 (Group 1), 10 μ g (Group 2), 50 μ g (Group 3), 100 μ g (Group 4) of ECMS or 50 μ g of Al-gel (Group 5) on the mean body weight (mean \pm SD gram) of mice ($n = 6$)

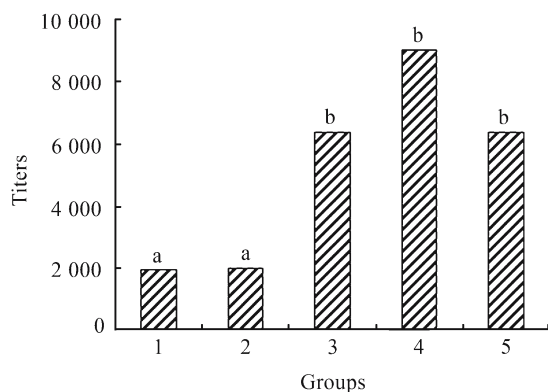
| Group | Before immunization* | Before booster* | 2 weeks after booster* |
|-------|----------------------|-----------------|------------------------|
| 1 | 19.7 \pm 1.07 | 21.6 \pm 0.96 | 22.3 \pm 1.15 |
| 2 | 20.6 \pm 1.55 | 21.2 \pm 1.53 | 22.0 \pm 1.92 |
| 3 | 20.4 \pm 0.27 | 22.2 \pm 0.64 | 23.0 \pm 0.56 |
| 4 | 20.5 \pm .83 | 22.3 \pm 0.31 | 22.8 \pm 0.26 |
| 5 | 20.5 \pm 0.94 | 21.7 \pm 1.37 | 22.5 \pm 1.37 |

*: No significance between groups at the same time ($P > 0.05$)

3.4 Adjuvant properties of ECMS in mice

3.4.1 Effect of ECMS on OVA-specific IgG response

There was a dose-dependent relation between ECMS and IgG titers (Fig. 2). The titers of OVA-specific IgG were positively proportional to the amount of ECMS co-administered. The titer in group 3, 4 or 5 injected with OVA solution containing 50 μ g or 100 μ g of ECMS or 50 μ g of Al-gel was significantly higher than that of the control (Group 1) ($P < 0.01$). The IgG titer in group 4 injected with OVA solution containing 100 μ g of ECMS was considerably high.



Six Balb/c mice in each group were immunized subcutaneously at a 3-week interval with 10 μ g of OVA mixed with 0 (Group 1), 10 μ g (Group 2), 50 μ g (Group 3), 100 μ g (Group 4) of ECMS or 50 μ g of Al-gel (Group 5). Blood samples were collected two weeks after the booster for separation of serum, which was used to determine specific-OVA IgG titers by using an ELISA. Bars with different letters show significant differences between groups ($P < 0.05$).

Fig. 2 Adjuvant effect of ECMS and Al-gel on IgG responses to OVA in mice

3.4.2 Effect of ECMS on OVA-specific IgG₁ and IgG_{2a} responses

The IgG₁ level in the serum from Group 4 injected with OVA solution containing 100 μ g of ECMS was significantly higher than that of the control (Group 1) and Group 2 ($P < 0.05$), but no significantly increased IgG₁ level in the sera from other groups was found when compared with that from the control ($P > 0.05$) (Fig. 3). The IgG_{2a} levels in sera from mice injected with OVA solution containing 10 μ g (Group 2) or 50 μ g (Group 3) or 100 μ g (Group 4) of ECMS or 50 μ g of Al-gel (Group 5) were significantly higher than that of the control (Group 1) ($P < 0.05$) with the highest IgG_{2a} level recorded in Group 3.

3.4.3 Effect of ECMS on splenocyte proliferation

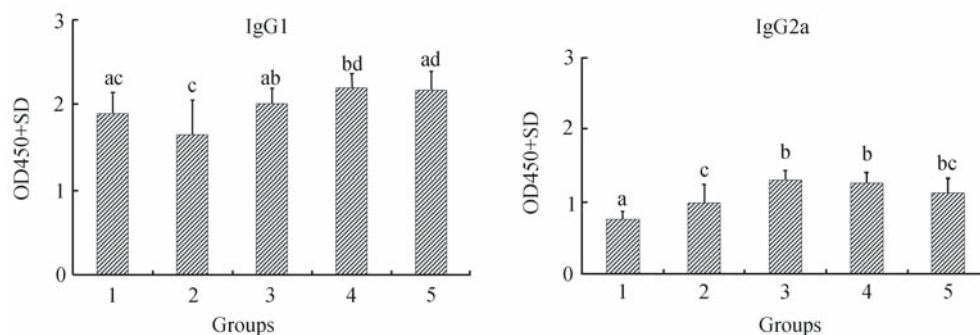
Figure 4 indicates that co-administration of OVA with ECMS significantly increased the splenocyte proliferative responses to Con A, LPS or OVA as compared with that co-administered with Al-gel or the control (saline group) ($P < 0.05$).

4 Discussion

An important finding in this research is the adjuvant effect of ECMS in mice. When co-administered with ECMS, OVA as a model antigen induced significantly higher specific antibody production than the OVA alone did (Fig. 2). Such adjuvant effect was dose-dependent: when ECMS was at a lower dose (10 μ g) no adjuvant effect was found, while when the dose of ECMS increased to 50 μ g or 100 μ g, OVA-specific IgG titer significantly increased. Such a dose-dependent mode was also found in other saponins such as ginsenosides (Rivera et al., 2003). When compared to Al-gel, ECMS showed a similar adjuvant potency as the mice produced almost the same antibody level when they were immunized with 10 μ g of OVA adjuvanted with 50 μ g of ECMS or the same amount of Al-gel (Fig. 2).

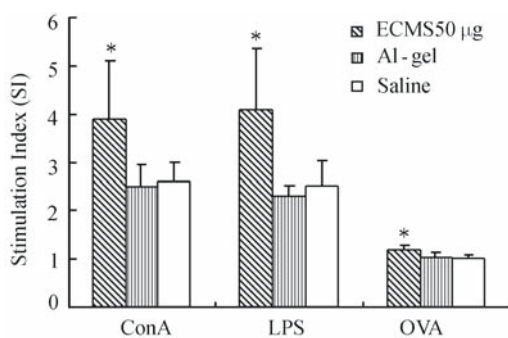
Our study also demonstrated that ECMS and Al-gel can promote the production of both IgG₁ and IgG_{2a} while Al-gel favored the production of IgG₁ and ECMS favored the production of IgG_{2a} (Fig. 3). In mice, cytokines such as interleukin-4 (IL-4), IL-5 and IL-10 produced by Th2 lymphocytes improved the production of IgG₁ while IL-2, tumor necrosis factor- β (TNF- β) and interferon- γ (IFN- γ) produced by Th1 lymphocytes improved the production of IgG_{2a} (Soren et al., 2000; Cherwinski et al., 1987). For the immunity against infectious diseases, Th1 response primarily targets the intracellular pathogens such as virus and some bacteria while Th2 response is mainly responsible for the extracellular pathogens such as most bacteria and parasites (Soren et al., 2000; Cherwinski et al., 1987). Thus, when a vaccine is designed to activate a higher Th1 than Th2 immune response, the ECMS is a more suitable adjuvant than Al-gel.

The splenocyte proliferative responses varied with the mitogen used. Both Con A- and LPS-induced proliferations



Six Balb/c mice in each group were immunized subcutaneously at a 3-week interval with 10 μ g of OVA mixed with 0 (Group 1), 10 μ g (Group 2), 50 μ g (Group 3), 100 μ g (Group 4) of ECMS or 50 μ g of Al-gel (Group 5). Blood samples were collected two weeks after the booster for separation of serum, which was used to determine specific-OVA IgG₁ and IgG_{2a} levels by using an ELISA. Bars with different letters mean a significant difference between groups ($P < 0.05$).

Fig. 3 Adjuvant effects of ECMS and Al-gel on the subclass IgG responses in mice



Six Balb/c mice in each group were immunized subcutaneously at a 3-week interval with 10 μ g of OVA mixed with saline or 50 μ g of ECMS or Al-gel. Splenocytes were collected two weeks after the booster, which was used to assay cell proliferative responses to Con A and LPS and OVA by using a MTT method as described in the text. Data were expressed as stimulation index (SI) and bars with different letters means a significant difference between groups ($P < 0.05$).

Fig. 4 Effects of ECMS and Al-gel on splenocyte proliferative responses to Con A, LPS and OVA in mice

were enhanced in the ECMS group. The enhanced lymphocyte response to Con A indicated that T lymphocytes were activated while the enhanced response to LPS indicated that B lymphocytes were activated. Although the splenocyte response to OVA antigenic stimulation in all the three groups was lower, the proliferation in the ECMS group was statistically higher than that in both saline and Al-gel groups. This result paralleled the increased antibody response detected in the mice injected with OVA co-administered with ECMS.

Results from this study demonstrate that ECMS is safe. Although Quil A has been reported to have adjuvant effect stimulating both humoral and cellular branches of the immune system (Windmill and Lee, 1999), its use as an adjuvant is limited due to its highly hemolytic activity (Sjølander and Barr, 1998). The ECMS used in this study showed lower hemolytic effect on RBC of swine, sheep and cattle than Quil A. No local reactions and negative effects on the body weight gain were found after injection of OVA mixed with various amount of ECMS in mice as compared with injection of OVA only.

Although this study reports the adjuvant effect of the ECMS in mice experiments, the ECMS used as a co-adjuvant for commercially available oil-adjuvanted vaccines for cattle has been evaluated. Those experiments show that the addition of ECMS to oil-adjuvanted vaccines may improve the immune responses of dairy cattle to the vaccination against foot and mouth disease.

In summary, when co-administered with ECMS in Balb/c mice, OVA can induce significantly higher production of IgG, IgG₁ and IgG_{2a}, but favor IgG_{2a}, and enhance splenocyte proliferative responses to Con A, LPS or OVA. The ECMS has low hemolytic activity and is safe for local injection. Therefore, the ECMS presented in this paper deserves further studies to evaluate its use in the development of new vaccines.

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