Full Length Research Paper

Enhancement of the immune responses to ovalbumin in mice by oral administration of the extract from 
*Radix cyathulae* (RC)

Haibo Feng¹,²#, Xiaogang Du¹#, Xiaohan Cao¹, Zhenhua Wang¹,³, Jing Tang¹, Juan Liu², Bing Zhao¹, Zhiyu Chen¹ and Xianyin Zeng¹*

¹Isotope Research Laboratory, College of Life and Physical Science, Sichuan Agricultural University, Ya’an, Sichuan 625014, PR China.
²Department of Veterinary Medicine, Southwest University, Rongchang, Chongqing, 402460, PR China.
³Department of Animal and Veterinary Science, Chengdu Vocational College of Agricultural Science and Technology, Wen Jiang, Sichuan 611130, P. R. China.

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This study was designed to evaluate the effects of oral administration of the extract from the *Radix cyathula* (RC) on the immune responses in mice immunized with ovalbumin (OVA). Forty-two Institute of Cancer Research (ICR) mice were randomly divided into six groups with seven animals in each group, and orally administered daily for 4 days at a dose equivalent to 0, 0.05, 0.1, 0.2, 0.4 and 0.8 g of the RC extract, respectively. After that, the mice were subcutaneously immunized twice with OVA at 2 weeks intervals. On day 14 after the second immunization, OVA-specific Immunoglobulin G (IgG) and the IgG subclasses, lymphocyte proliferation response, as well as interleukin-4 (IL-4) and interferon-gamma (IFN-γ) production were measured. Results indicated that the optical density (OD) value of OVA-specific IgG and the IgG subclass were significantly enhanced in mice orally administered RC at the dose of 0.2, 0.4 or 0.8 g, compared to the control group (*p* < 0.05). Besides, the splenocytes proliferation response and the production of IL-4 and IFN-γ were also significantly improved (*p* < 0.05) after the splenocytes were stimulated by OVA. In addition, the body weight of the mice administered with the RC extract (0, 0.1, 0.2, 0.4 and 0.8 g) was not significantly different compared to the mice administered saline. Collectively, the data suggested that RC extract could improve the immune response of vaccine through up-regulating the humoral immune response and the cellular immune response.

Key words: *Radix cyathula*, ovalbumin, adjuvant.

INTRODUCTION

Vaccination is the most cost-effective approach for the control of infectious diseases. The traditional vaccines, typically including the live attenuated or inactivated whole organisms, have been very successful in inducing immune response. However, the safety of the traditional vaccines is questionable due to the risk of the virulence reversion (Lima et al., 2004). But, the new generation vaccines such as purified recombinant proteins, synthetic peptides and plasmid DNA, are unfortunately often much less reactogenic and immunogenic (Hagan et al., 2001). Many strategies are used to improve the immune response to vaccination including the use of higher vaccine

*Corresponding author. E-mail: xyzeng1966@163.com or xyzeng@sicau.edu.cn. Tel: +86 835 2886136, Fax: +86 835 2886136.

These authors contributed equally to the work.
dose, increasing number of doses, the different route of administration (for example, intradermal versus intramuscular administration), accelerating dosing schedule and adjuvants such as antigen delivery systems and various immune modulators (Rawer et al., 1987; Donati and Gastaldi, 1988; Quiroga et al., 1990; Bryan et al., 1992; Jungers et al., 1994; Taglietti, 1995; Disis et al., 1996; Mitwalli et al., 1998; Rey et al., 2000). Among them, using the adjuvants maybe is a feasible way to increase the potency on the appropriate immune response (Rock et al., 2005; Storni et al., 2005).

There are many growing evidences that medicinal herbs and the ingredients can enhance the immune response to vaccines direct against infectious agents (Liu et al., 1992; Rajput et al., 2007). It has demonstrated that the co-administration of vaccines and herbal extracts has increased the antibody response and the T cell proliferative response (Hu et al., 2003; Sun et al., 2007). Several herbal extracts as adjuvant of vaccines have been used in the human and animal clinical trials (Scagliesi et al., 1990; Scagliesi et al., 1994; Scagliesi et al., 1996).

_Cyathula officinalis_ (Amaranthaceae family) is a perennial herbaceous plant widely distributed in tropical areas of Asia and Africa, particularly in China, Korea and Vietnam. Its root ("Chuan Niu X" in Chinese, _C. officinalis kuan_) is a kind of commonly-used Chinese traditional herbal medicine with a wide range of pharmacological activities (National Pharmacopoeia Committee, 2005a). It is usually used as a tonic, emmenagogue, antiarthritic, diuretic, and antifebrility agent to nourish the liver and kidneys, strengthen bones and muscles, and invigorate circulation (National Pharmacopoeia Committee, 2005b). The modern pharmacological studies demonstrated that the root of _C. officinalis_ possessed many functions such as immunostimulant (Ye et al., 2007; Wang et al., 2007), uteri-excitant and antifebrility (Li et al., 1990; Li et al., 1998), antitumor (Song, 1994; Chen et al., 2003a), analgesic, antibacterial, anti-inflammatory (Song and Yang, 2001), antisenile (Li et al., 1998). However, to date, _Radix Cyathulae officinalis_ Kuan (RC) as an adjuvant for vaccines has not yet been reported. In the present study, we attempted to evaluate the enhancement of the immune responses to ovalbumin (OVA) in mice by oral administration of the RC extract. In this study, we demonstrated for the first time that RC can efficiently enhance the immunogenicity of OVA via improving the humoral and cellular immune responses and the cytokine expression.

**MATERIALS AND METHODS**

**Animals**

Female Institute of Cancer Research (ICR) mice (Grade II, 5 weeks old) were purchased from Sichuan Laboratory Animal Center (SLAC) Co. Ltd. (Sichuan, China), and housed in polypropylene cages with sawdust bedding in hygienically controlled environment. Food and water were supplied ad libitum. The temperature was gradually declined to the room temperature and the light time is up to 12 h/day. All procedures related to the animals and their care conformed to Guidelines for Keeping Experimental Animals issued by the government of China.

**Materials**

OVA, concanavalin A (ConA), lipopolysaccharide (LPS), RPMI-1640 medium, and goat anti-mouse Immunoglobulin G (IgG) peroxidase conjugated were purchased from Sigma Chemical Co., Ltd (Saint Louis, Missouri, USA). The tachypleus amebocyte lysate (TAL) (sensitivity: λ = 0.125 EU/ml) was bought from Zhanjiang A&C Biological Ltd. (Zhanjiang, China). The control standard endotoxin (CSE) and water for the bacterial endotoxin test (BET) were provided by National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Cell counting kit-8 (CCK-8) was obtained from Beyotime institute of Biotechnology Haimen (Jiangsu, China). The goat anti-mouse IgG, IgG1, IgG2a and IgG2b conjugated with the peroxidase were gotten from Santa Cruz Biotechnology Inc (California, USA). Enzyme-linked immunosorbent assay (ELISA) kits of detecting interleukin-4 (IL-4) and interferon-gamma (IFN-γ) were from Wuhan Boster Biological Technology Co. Ltd (Hubei, China). Fetal calf serum (FCS) was provided by Invitrogen Gibco Biotechnology Inc (California, USA).

**Preparation of RC extract**

Dried RC was purchased from Tong Ren Tang Co. Ltd (Ya’an, China). After cleaning and drying, RC was cut into pieces, and ground into powder. Then, 100 g of RC powder was saturated in 1000 ml of water. After that, the supernatant was collected by passing through a filter paper and condensed at a concentration equivalent to 4 g of dried RC per ml by a R502B rotary evaporator (Shenko Tech Co. Ltd., Shanghai, China). The diluted preparations were sterilized by pasteurization, and the endotoxin content was determined using the Limulus amebocyte lysate test (National Pharmacopoeia Committee, 2005). When the endotoxin level in RC extract solution was less than 0.5 EU/ml, it was stored at 4°C for use in the future.

**Oral administration of RC extract and immunization**

Forty-two ICR mice were randomly divided into six groups (n = 7). The animals were subcutaneously injected twice with 100 µg of OVA with 2 weeks intervals, as described previously (Li et al., 2009). Before each immunization, the mice had been orally administered daily for 4 days with 0.25 ml of 0.9% saline solution containing RC of 0, 0.05, 0.1, 0.2, 0.4 or 0.8 g (equivalent to 0, 2.05, 4.1, 8.2, 16.4 or 32.8 g/kg bodyweight).

**Enzyme-linked immunosorbent assay (ELISA)**

Blood samples were collected at 2 weeks after the boost, the optical density (OD) value of OVA-specific IgG and isotopes were measured by an indirect ELISA as described by Du (2007). Briefly, the microtiter plate wells were coated with 100 µl of OVA at 50, 25, 50 and 50 µg/ml for testing IgG, IgG1, IgG2a and IgG2b antibodies overnight at 4°C. After three washes, the wells were blocked with 5% skimmed milk and incubated for 1 h at 37°C. Followed by three times of washing, 100 µl of serum (diluted 1:50 in phosphate-buffered saline (PBS) 5% skimmed milk) was added to each well and incubated at 37°C for 1 h. The horseradish peroxidase-conjugated antibodies against IgG, IgG1, IgG2a or IgG2b were incubated for 1 h at 37°C. After washing, the peroxidase activity was assayed as following: the substrate solution (10 mg of O-
Figure 1. OVA-specific IgG. Mice were orally administered with RC solution (0, 0.05, 0.1, 0.2, 0.4 or 0.8 g of RC/mouse/day) for 4 days, and then immunized s.c. twice with 100 µg of OVA at 2 weeks intervals. Blood samples were collected at the second week after boost for testing the OD value of OVA-specific IgG by an indirect ELISA. The OD values are presented as mean ± SD (n = 7). Bars with different letters are statistically different (p < 0.05).

phenylenediamine and 37.5 µl of 30% H2O2 in 25 ml of 0.1 M citrate–phosphate buffer, pH 5.0) was added to each well, and incubated for 10 min at 37°C. The reaction was stopped by 2 M H2SO4. The OD was measured in an ELISA reader at 450 nm. Data were expressed as the mean OD value of the samples minus the mean OD value of the blank control.

Splenocyte proliferation assay

Mice were sacrificed on day 14 after the boost, and spleen were collected from the mice under aseptic conditions. The spleen was minced and passed through a fine steel mesh to obtain a homogeneous cell suspension. Then, the erythrocytes were lysed with ammonium chloride solution (0.8%, w/v). After washed three times in PBS, the splenocytes were suspended in incomplete medium (RPMI 1640 supplemented with 0.05 mM 2-mercaptoethanol, 100 UI/ml penicillin, 100 µg/ml streptomycin and 10% heat inactivated FCS). The splenocytes were counted with the haemocytometer by the trypan blue dye exclusion technique. Splenocytes proliferation was performed as previously described (Sun et al., 2004). Briefly, splenocytes were seeded into four wells of a 96-well flat-bottom microtiter plate at 2.5 × 10^6 cell/ml in 100 µl complete medium, thereafter ConA (5 µg/ml), LPS (10 µg/ml), OVA (5 µg/ml), or medium were added giving a final volume of 200 µl. The plates were incubated in a humid atmosphere with 5% CO2 at 37°C. After 44 h, 20 µl of WST-8 solution (5 mg/ml) was added to each well and incubated for another 4 h. The OD at 450 nm was determined using a microplate reader. The stimulation index (SI) was calculated based on the following formula: SI = the absorbance value for the stimulation-cultures divided by the absorbance value for non-stimulated cultures.

IFN-γ and IL-4 production by spleocyte

The single splenocyte suspension was prepared on day 14 after the boost, and the splenocyte (2.5 × 10^6 cells/ml) were incubated with OVA (final concentration 5 µg/ml) in 96-well culture plates at 37°C in 5% CO2. After 48 h, the culture supernatant was collected for the detection of IL-4, and IFN-γ levels by the commercial ELISA kits according to the manufacturer’s instructions.

Statistical analysis

Data analysis was performed with SPSS software (SPSS, Version 11.5, SPSS Inc., Chicago, IL). Analysis of variance (ANOVA) with Bonferroni post-hoc test was used for multiple comparisons between groups. Values were expressed as the mean ± standard deviation (SD). p-Values of less than 0.05 were considered statistically significant.

RESULTS

Humoral immune response

To evaluate the adjuvant effect of RC on the humoral immune responses, the OD value of the antigen specific IgG and the IgG subclasses were measured by the indirect ELISA. As shown in Figure 1, the OD values of IgG were significantly higher in mice orally administered RC at a dose of 0.05, 0.1, 0.2, 0.4 and 0.8 g than that in the saline group (Figure 2). But, the significantly increased OD values of IgG and isotypes were observed in mice administered RC at doses of 0.4 and 0.8 g (p < 0.05).

Splenocytes proliferation response

To detect the effects of RC on splenocyte proliferative responses, the proliferation assay was performed by the WST method. As shown in Figure 3, SI of the proliferation response to ConA, LPS and OVA was gradually increased in mice administered at 0.2, 0.1 and 0.05 g of RC, and significantly increased in mice administered at
Figure 2. OVA-specific IgG isotypes. Mice were orally administered RC solution (0, 0.05, 0.1, 0.2, 0.4 or 0.8 g of RC/mouse/day) for 4 days, and then immunized s.c. twice with 100 µg of OVA at 2 weeks intervals. Blood samples were collected at the second week after the boost for testing OD values of OVA-specific IgG isotypes by an indirect ELISA. The values are presented as mean ± SD (n = 7). Bars with different letters are statistically different (p < 0.05).

Figure 3. Splenocyte proliferative responses. Mice were orally administered RC solution (equivalent to 0, 0.05, 0.1, 0.2, 0.4g or 0.8 g of RC/mouse/day) for 4 days, and then immunized s.c. twice with 100 µg of OVA at 2 weeks intervals. Splenocytes were prepared at second week after the boost and cultured with ConA (5 µg/ml) or LPS (10 µg/ml) or OVA (5 µg/ml) or RPMI-1640 for 48 h. Splenocytes proliferation was measured by the WST-8 method as described in the work and shown as a stimulation index (SI). The values are represented mean ± SD (n = 7). Bars with different letters are statistically different (p < 0.05).

Figure 4A. IL-4 production was significantly higher in the mice administered RC (0.8 and 0.4 g) than that in the mice administered saline solution (p < 0.05). Moreover, IFN-γ production was significantly higher in the group administered RC (0.8, 0.4, 0.2 and 0.1 g) than that in the group administered saline solution (Figure 4B).

Effects of RC extract on IL-4 and IFN-γ

To test, RC extract influences the cytokine secretion, IL-4 and IFN-γ production was measured by ELISA. As shown in Figure 4A, IL-4 production was significantly higher in the mice administered RC (0.8 and 0.4 g) than that in the mice administered saline solution (p < 0.05). Moreover, IFN-γ production was significantly higher in the group administered RC (0.8, 0.4, 0.2 and 0.1 g) than that in the group administered saline solution (Figure 4B).
Effects of RC on the body weight of mice

To evaluate the safety of RC extract, the body weight of mice was measured before and after the first immunization and boost. No abnormal behavior and side effects were observed in mice throughout the experiment. Besides, there was no significant difference in the body weight between the mice administered RC (0, 0.05, 0.1, 0.2, 0.4 or 0.8 g) and the control group (Table 1) ($p > 0.05$).

DISCUSSION

An efficient adjuvant should have the negligible toxicity and elicit the robust humoral or/and cellular immune response against the specific antigen (Marciani et al., 2003). While several hundred different adjuvants, including mineral salts, microorganism-derived adjuvants, cytokines, emulsions and polysaccharides, have been tested for the research or usage in novel vaccine design over the last few decades, the vast majority of the adjuvants have not been successfully approved for human use, because of the unacceptable local or systemic toxicity reaction (McCluskie and Weeratna, 2001; Spickler et al., 2003; Aguilar and Rodriguez, 2007).

Many natural products have been reported having immunomodulatory properties, while their active ingredients are not clear (Banji et al., 2012). It is difficult to purify the herbal extracts, because the co-injection with the unpurified herbal extracts and antigens can induce the irritation (Qi et al., 2011). The traditional medicinal herbs are administered by oral route, because the oral use of immunomodulators can avoid the side effects elicited through the parenteral administration (Hu et al., 2011). For example, oral administration of Rhizoma
Atractylodis Macrocephalae extract can significantly increased the immune responses against foot-and-mouth disease in mice, no abnormal behavior and side effects were found in mice throughout the experiment (Li et al., 2009). Maharaj et al. (1986) and Chavali et al. (1987) reported that the crude saponin from the bark of the Quillaja tree by the oral administration had the immunopotentiating activity with moderated toxicity, however, they were toxic when administered parenterally.

The cellular immune response plays an important role in the host response against the intracellular pathogens via limiting their replication and accelerating clearance of the infected cells (Arama et al., 2012). Among T-lymphocytes, the helper T-cells induces B-lymphocytes to secrete antibodies, and the cytotoxic T-lymphocytes help phagocytes to destroy ingested microbes and to clear the intracellular microbes. However, the humoral immunity mediated by antibodies, neutralize and eliminate the extracellular microbes and microbial toxins (Montomoli et al., 2011). The capacity to elicit the effective T- and B-lymphocyte immunity can be shown by the lymphocyte proliferation response (Effros, 2007). It is generally known that ConA stimulates T-cells and LPS stimulates B-cell proliferation. In this study, RC promoted the ConA-, LPS- and OVA-stimulated splenocyte proliferation in the immunized mice (Ren et al., 2012). It indicates that RC can significantly improve the humoral immunity and cell-mediated immune response via increasing the activation potential of T- and B-cells. Similar results have been found in other studies. Jia et al. (2009) reported that oral administration of the extract from RC for 10 days significantly enhanced lymphocyte transformation in response to ConA stimulation in mice. Xiang et al. (1994) have demonstrated that the lymphocyte proliferation in response to ConA and LPS stimulation and natural killer (NK) cell activity were enhanced in rats fed by a diet supplemented with the RC powder.

The cytokines have the pivotal function in regulating the immune response. Th2 lymphocyte cytokines such as IL-4, IL-5 and IL-10 can augment the IgG1 production, while IL-2, TNF-α and IFN-γ produced by Th1 lymphocytes can improve IgG2a production (Chavali et al., 1987; Soren et al., 2000). Oral administration of RC significantly increased the level of IgG1, IgG2a and IgG2b (Figure 2). In terms of the host response against the infectious diseases, Th1 response primarily targets the intracellular pathogens such as viruses and the certain bacteria, but Th2 response mainly targets the extracellular pathogens, such as most bacteria and certain parasites (Chavali et al., 1987; Soren et al., 2000). The higher level of all IgG subclasses may be explained by increasing both IL-4 and IFN-γ production (Figure 4), suggesting that RC can enhance both Th1 and Th2 immune responses.

In addition to the immunomodulatory effects, RC has the anti-inflammatory activity as well. RC together with other herbs has been used to treat the inflammatory reaction in the human upper respiratory tract (Ye et al., 2007). Shi and Zheng, (1988) have found that the OVA-induced the acute inflammatory edema of the foot in rats was significantly reduced after the oral administration of the compounds such as polysaccharide extracted from the RC. Imbalance of Th1/Th2 lymphocytes may be one of the reasons for some inflammatory diseases (Smart et al., 2002). In this study, RC has been found to stimulate both IFN-γ (Th1-like cytokine) and IL-4 (Th2-like cytokine), suggesting that RC can promote the balanced Th1/Th2 response. This may constitutes the anti-inflammatory mechanism of RC.

Adjuvant should be safe enough to induce the minimal adverse effects for use in human and animals. In this study, no abnormal behaviors and side effects were observed in mice throughout the experiment, and there was no significant difference in the body weight between the mice administered RC and the control mice (Table 1). It suggests that oral administration of RC is safe.

Table 1. Effects of oral administration of RC on the mean body weight (mean ± S.D, n = 7).

<table>
<thead>
<tr>
<th>Gram of RC/mouse</th>
<th>Before first immunization</th>
<th>Before boost</th>
<th>2 Weeks after boost</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>22.62 ± 0.62</td>
<td>26.08 ± 0.28</td>
<td>26.89 ± 0.57</td>
</tr>
<tr>
<td>0.05</td>
<td>23.00 ± 0.96</td>
<td>27.10 ± 0.73</td>
<td>27.62 ± 0.52</td>
</tr>
<tr>
<td>0.1</td>
<td>22.37 ± 0.16</td>
<td>26.51 ± 0.42</td>
<td>26.97 ± 0.96</td>
</tr>
<tr>
<td>0.2</td>
<td>23.06 ± 0.31</td>
<td>26.66 ± 0.45</td>
<td>27.14 ± 0.70</td>
</tr>
<tr>
<td>0.4</td>
<td>23.22 ± 0.14</td>
<td>25.82 ± 0.55</td>
<td>27.40 ± 0.46</td>
</tr>
<tr>
<td>0.8</td>
<td>22.92 ± 0.69</td>
<td>26.28 ± 1.05</td>
<td>27.08 ± 0.75</td>
</tr>
</tbody>
</table>
proliferation response as well as production of IL-4 and IFN-γ in mice immunized with OVA. Considering the immunomodulatory effect and safety of RC demonstrated in this study, this herb as an adjuvant deserves further investigation of its potential on improving the immune response in other animal models such as pigs, goats and cattle in the future.

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REFERENCES


