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Immunological Adjuvant Activity of Two Chinese Medicine Prescription Preparations

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Abstract: The aim of this study was to evaluate and compare adjuvant potential of two widely used Chinese medicine prescription preparations Golden Screen health Capsules (GSC) and Yupingfeng Koufuye (YPF) on immune responses to Ovalbumin (OVA) in mice. Mice were administered orally with GSC or YPF at the different doses for 5 days and then immunized subcutaneously twice with OVA 100 μg alone at 2 weeks intervals. The 2 weeks later, splenocyte proliferation, Natural Killer (NK) cell activity, production of cytokines from splenocytes and serum OVA-specific antibody titers were measured. GSC and YPF significantly not only the serum OVA-specific IgG and IgG1 antibody titers but remarkably enhanced Con A-LPS and OVA-induced splenocyte proliferation and NK cells activities in OVA-immunized mice. The production of cytokine IL-2 from splenocytes and serum OVA-specific IgG2a and IgG2b antibody titers in the immunized mice were also significantly enhanced by YPF. YPF was better than GSC and alum in the quality and strengths to enhance specific cellular and humoral immune responses against OVA in mice with YPF preferred for those situations where mixed Th1/Th2 responses are needed.

Key words: Golden Screen health Capsules (GSC), Yupingfeng Koufuye (YPF), adjuvant, oral administration, cellular and humoral immune responses

INTRODUCTION

Vaccination is regarded as one of the most cost-effective preventative healthcare measures. Many conventional vaccines are made of attenuated or killed pathogens thus containing naturally a number of signals able to activate the innate immune response. However, a growing number of more recent vaccines are made of recombinant proteins devoid of such signals and therefore poorly immunogenic. In developing an effective vaccine, besides the primary challenge of identifying the most relevant immunogen and efficient regime of immunization, selection of a potent adjuvant is equally critical. The use of novel adjuvants in combination with novel immunogen design holds great promise towards the goal of enhancing the potency, breadth and durability of vaccines (Dey and Srivastava, 2011).

A number of substances with documented adjuvant activity have been reported over the last 20 years. A key issue in adjuvant development is the minimization of toxicity while maintaining effectiveness (Coffman *et al*., 2010). Owing to safety concerns, very few adjuvants have been licensed for use in human vaccines (Tritto *et al*., 2009). Alum was first applied as an adjuvant >80 years ago (Glenny *et al*., 1931). Until recently alum was the only licensed adjuvant in the USA (Exley *et al*., 2010). However, Alum fails to confer adequate increase of antibody response to small-size peptides as well as certain vaccines such as typhoid fever and influenza vaccines. Notably, alum is known to be a poor adjuvant for induction of cytotoxic T cell immunity and T helper 1 (Th1) responses, required to combat several life-threatening infections (Exley *et al*., 2010). In addition, its use presents several drawbacks: alum preparations are heterogeneous; they can produce granulomas at the injection site and vaccines formulated with alum may become ineffective when frozen, a feature that constitutes a serious problem, mainly in third world countries (Reed *et al*., 2009). Together with constraints in terms of the type of response generated by alum, these limitations have led to the search for new adjuvants for human vaccines (Harandi *et al*., 2010).

Plant-derived medicines have a long history of use for the prevention and treatment of human disease. Today, many pharmaceuticals currently approved by the Food and Drug Administration (FDA) have origins to plant sources (Licciardi and Underwood, 2011). Many widely used traditional Chinese medicines and their

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ingredients were claimed to enhance the immune response to vaccines direct against infectious agents (Sun *et al*., 2009a; Granell *et al*., 2010; Petrovsky and Cooper, 2011). Their efficacy as adjuvant to vaccines has also been appraised in both humans and animals (Quan *et al*., 2007; Underwood *et al*., 2009; Licciardi and Underwood, 2010). However, very few have been proven to have efficacy by controlled experiments and found their way into the clinic (Gertsch *et al*., 2011). Therefore, there is still suspicion concerning the efficacy and optimum dosage of plant-derived medicines and their ingredients as adjuvants (Hashemi and Davoodi, 2012).

The traditional Chinese medicines are usually administered orally in the form of formulas. It is regarded that the great majority of traditional Chinese medicine is more effective than the individual components. The irritation will take place when unpurified extracts are co-injected with immunizing antigens. However, the purification of the components in the traditional Chinese medicines is usually difficult. Therefore, the oral use of these medicines as adjuvants can avoid the side effects found in parenteral administration.

Ovalbumin (OVA) is commonly used as the model antigen to detect the adjuvant effect of chemicals (Larsen and Nielsen, 2007). Researchers have earlierly used OVA as the model antigen to screen the saponins and polysaccharides with the adjuvant properties from a wide range of Chinese herbal medicines (Xie *et al*., 2008a; Sun *et al*., 2009b). The obtained ingredients with the adjuvant properties have also been verified their adjuvant effects on recombinant hepatitis B surface vaccine and Newcastle disease virus-based recombinant avian influenza vaccine in the mice and chickens (Xie *et al*., 2009, 2010). To scientifically validate traditional Chinese medicines as adjuvants after oral ingestion, in this study, two widely used and commercially available Chinese medicine prescription preparations Golden Screen health Capsules (GSC) and Yupingfeng Koufuye (YPF) have been selected based on prevalence of use and reports describing immunomodulatory activity (Zhang *et al*., 2000; Liu *et al*., 2009). Using an approach that combined parenterally delivered antigen OVA and the mucosal administration of adjuvant, the antigen-specific cellular and humoral immune responses of mice was investigated following oral consumption of GSC and YPF with well-acknowledged vaccine adjuvants Alum and Quil A as the positive control.

MATERIALS AND METHODS

Chemicals and reagents: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Concanavalin A (Con A), Lipopolysaccharide (LPS), RPMI-1640 medium and rabbit anti-mouse IgG peroxidase conjugate were purchased from Sigma Chemical Co., St. Louis, MO; goat anti-mouse IgG1, IgG2a and IgG2b peroxidase conjugate were from Southern Biotech. Assoc., Birmingham, AL; cytokine (IL-2) detecting ELISA kits were from Wuhan Boster Biological Technology Co., Ltd. Hubei, China. Quil A was kindly provided by Brenntag Nordic A/S, Denmark. Fetal Calf Serum (FCS) was provided by Hangzhou Sijiqing Corp., Hangzhou, Zhejiang, China; Aluminum hydroxide gel (Alum) was from Zhejiang Wanma Pharm Co., Ltd. Hangzhou, Zhejiang, China. Human leukemia K562 cell lines, sensitive to Natural Killer (NK) cells were purchased from Institute of Cell Biology, Chinese Academy Sciences. They were maintained in the logarithmic phase of growth in RPMI-1640 medium supplemented with 2 mM L-glutamine, 100 IU mL^{-1} penicillin, 100 μg mL^{-1} streptomycin and 10% FCS at 37°C under humidified air with 5% CO_2.

Experimental animals: Female ICR mice (Grade II, 5 weeks old) weighing 18-22 g were purchased from Zhejiang Experimental Animal Center (Certificate No. 22-2001001, Hangzhou, China) and acclimatized for 1 week prior to use. Rodent laboratory chow and tap water were provided *ad libitum* and maintained under controlled conditions with a temperature of 24±1°C, humidity of 50±10% and a 12/12 h light/dark cycle. All the procedures were in strict accordance with the PR China legislation on the use and care of laboratory animals and with the guidelines established by Institute for Experimental Animals of Zhejiang University and were approved by the university committee for animal experiments.

Preparation of golden screen health capsules and Yupingfeng Koufuye: Golden Screen health Capsule (Jinpingfeng capsule, GSC) is comprised of Echinacea purpurea extracts, Astragalus membranaceus extracts and vehicles starch. The 100 g of GSC contains the astragaloside 1350 mg and cichoric acid 150 mg. Yupingfeng Koufuye (YPF) is prepared from three traditional Chinese drugs Radix Astragali, Rhizoma Atractylodis Macrocephalae (fried) and Radix Saposhnikoviae. The 1000 mL of YPF contains Radix Astragali 600 g, Rhizoma Atractylodis Macrocephalae 200 g and Radix Saposhnikoviae 200 g. The GSC and YPF used in this study were produced from Chiatai Qingchunbao Pharmaceutical Co., Ltd. Hangzhou and Zhejiang Xinguang Pharmaceutical Co., Ltd. Shengzhou, Zhejiang, China, respectively. Both GSC and YPF are commercially available Chinese medicine prescription preparations which meet current standards required for

human use. The doses administered orally per mouse was 2.5, 5, 10 and 20 mg (equivalent to preparation/day) for GSC and 0.0625, 0.125, 0.25 and 0.5 g (equivalent to crude drug/day) for YPF, respectively based on the proportional daily human consumption for a 60 kg human and calculated on the daily mouse fluid consumption rates previously determined. The GSC solution was prepared by dissolving in 0.89% saline and YPF was diluted with saline into the suitable concentrations.

Oral administration and immunization: The 6 weeks old female ICR mice were divided into 12 groups each consisting of six mice. Animals were immunized subcutaneously with OVA 100 μg alone or with OVA 100 μg dissolved in saline containing alum (200 μg) or Quil A (10 μg) on day 1. The mice were orally administered daily with saline or GSC in saline at a single dose of 2.5, 5, 10 and 20 mg (equivalent to preparation) or YPF diluted in saline at a single dose of 0.0625, 0.125, 0.25 and 0.5 g (equivalent to crude drug) per mouse for 5 days prior to immunization with OVA alone. Saline-treated animals were included as blank controls. A boosting injection was given 2 weeks later. Sera and splenocytes were collected 2 weeks after the second immunization for measurement of OVA-specific antibody and proliferation, NK cell activity and cytokine assay.

Splenocyte proliferation assay: Splenocytes from the OVA-immunized mice under aseptic conditions were prepared as earlierly described (Sun, 2006) and were seeded into three to four wells of a 96 well flat-bottom microtiter plate (Nunc) at 5×10^6 cells mL^{-1} in 100 μL complete medium, thereafter Con A (final concentration 5 μg mL^{-1}), LPS (final concentration 10 μg mL^{-1}), OVA (final concentration 20 μg mL^{-1}) 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. After 44 h, 50 μL of MTT solution (2 mg mL^{-1}) was added to each well and incubated for further 4 h. The plates were centrifuged (1400×g, 5 min) and the untransformed MTT was removed carefully by pipetting 150 μL of a DMSO (Sigma, St. Louis, MO) working solution (192 μL DMSO with 8 μL 1N HCl) was added to each well and the absorbance was evaluated in an ELISA reader at 570 nm after 15 min. The Stimulation Index (SI) was calculated based on the following equation:

$$SI = \frac{\text{The absorbance value for mitogen-cultures}}{\text{The absorbance value for non-stimulated cultures}}$$

Assay of NK cell activity: The activity of NK cells in splenocytes in the immunized mice was measured as previously described (Ouyang *et al*., 2012). K562 cells were used as target cells and seeded in 96 well U-bottom microtiter plate at 2×10^4 cells/well in RPMI 1640 complete medium. Splenocytes prepared as described above were used as the effector cells and were added at 1×10^6 cells/well to give E/T ratio 50:1. The plates were then incubated for 20 h at 37°C in 5% CO_2 atmosphere. The 50 μL of MTT solution (2 mg mL^{-1}) was added to each well and the plate was incubated for another 4 h and subjected to MTT assay. Three kinds of control measurements were performed: target cells control, blank control and effector cells control. NK cell activity was calculated as following equation:

$$\text{NK cell activity (\%)} = \frac{(OD_T-(OD_S-OD_E))}{OD_T}\times100\%$$

Where:
OD_T = Optical density value of target cells control
OD_S = Optical density value of test samples
OD_E = Optical density value of effector cells control

Measurement of OVA-specific antibody: OVA-specific IgG, IgG1, IgG2a and IgG2b antibodies in sera were detected in individual serum samples by an indirect ELISA (Xie *et al*., 2008b). In brief, microtiter plate wells were coated with 100 μL OVA solution (25 μg mL^{-1} in 50 mM carbonate-bicarbonate buffer, pH 9.6) for 24 h at 4°C. The wells were washed three times with PBS containing 0.05% (v/v) Tween 20 (PBS/Tween) and then blocked with 5% FCS/PBS at 37°C for 2 h. After three washings, 100 μL of a series of diluted sera sample or 0.5% FCS/PBS as control were added to triplicate wells. The plates were then incubated for 2 h at 37°C, followed by three times of washing. Aliquots of 100 μL of rabbit anti-mouse IgG horseradish peroxidase conjugate diluted 1:20,000, goat anti-mouse IgG1 peroxidase conjugate 1:8,000, IgG2a peroxidase conjugate 1:8000 and IgG2b peroxidase conjugate 1:8000 with 0.5% FCS/PBS were added to each plate. The plates were further incubated for 2 h at 37°C. After washing, the peroxidase activity was assayed as following: 100 μL of substrate solution (10 mg of O-phenylenediamine and 37.5 μL of 30% H_2O_2 in 25 mL of 0.1 M citrate-phosphate buffer, pH 5.0) was added to each well. The plate was incubated for 10 min at 37°C and enzyme reaction was terminated by adding 50 μL $well^{-1}$ of 2M H_2SO_4. The optical density was measured in an ELISA reader at 490 nm where sets of sera samples have been subjected to within and between group comparisons, ELISA assays were performed on the same day for all of the samples.

Cytokine measurements in the cultured supernatants of splenocytes by ELISA: Splenocytes (5×10^6 cells/well) from the immunized mice prepared as described before were incubated with Con A (final concentration 5 μg mL^{-1}) in 24 well culture plates at 37°C in 5% CO_2. After 48 h, the plate was centrifuged at 1400×g for 5 min and culture supernatants were collected for the detection of IL-2 levels using commercial ELISA kits. Briefly, culture supernatants or cytokine standards were added to 96 well flat-bottom microtiter plate coated with coating antibody and plates then incubated at 37°C for 1.5 h. Plates were washed and a detecting antibody was added to each well. Plates were incubated at 37°C for 1 h before addition of Avidin-Biotin-peroxidase Complex (ABC). After incubation for 30 min, plates were washed and developed with Tetramethyl Benzidine (TMB) at 37°C for 15 min. The reaction was stopped by addition of 100 μL of stop solution. The absorbance was measured in an ELISA reader at 450 nm.

Statistical analysis: The data were expressed as mean±SD and examined for their statistical significance of difference with ANOVA and a Tukey post hoc test. The $p<0.05$ were considered to be statistically significant.

RESULTS

Effects of oral administration of GSC and YPF on splenocyte proliferation in OVA-immunized mice: The effects of oral administration of GSC and YPF on mitogen and OVA-stimulated splenocyte proliferation in the mice immunized with OVA are shown in Fig. 1. Con A-stimulated splenocyte proliferation in the mice immunized with Quil A, GSC and YPF was significantly higher than that in the OVA alone group ($p<0.05$ or $p<0.01$). Quil A, GSC and YPF could also significantly promote LPS and OVA-induced splenocyte proliferation in the OVA-immunized compared with OVA alone group ($p<0.05$, $p<0.01$ or $p<0.001$). However, no significant differences ($p>0.05$) were observed between the OVA alone and OVA/Alum groups. These data indicated that oral administration of GSC and YPF were more effective than Alum in inducing strong activation potential of T and B cells in OVA-immunized mice.

Effects of oral administration of GSC and YPF on NK cell activity in OVA-immunized mice: The effects of oral administration of GSC and YPF on the activity of NK cell in splenocytes from the mice immunized with OVA were shown in Fig. 2. Quil A, GSC (10 and 20 mg) and YPF at four doses significantly enhanced the killing activity of

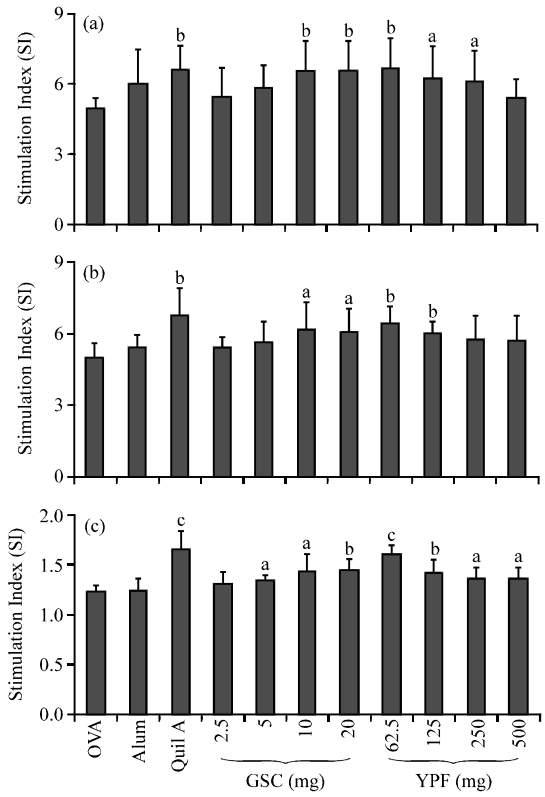

Fig. 1: Effect of oral administration of GSC and YPF on splenocyte proliferation in OVA-immunized mice. The values are presented as mean±SD (n = 6). Significant differences with OVA control group were designated as $^{a}p<0.05$, $^{b}p<0.01$ and $^{c}p<0.001$; a) Con A; b) LPS and c) OVA

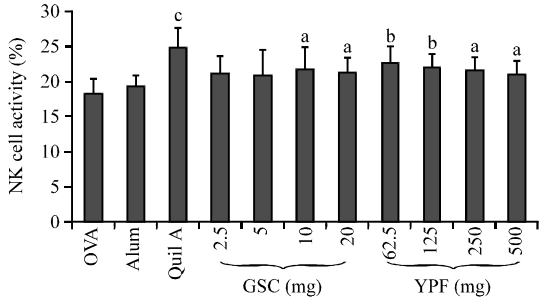

Fig. 2: Effect of oral administration of GSC and YPF on the activity of NK cell from splenocytes in OVA-immunized mice. The values are presented as mean±SD (n = 6). Significant differences with OVA control group were designated as $^{a}p<0.05$, $^{b}p<0.01$ and $^{c}p<0.001$

NK cell in the OVA-immunized mice ($p<0.05$, $p<0.01$ or $p<0.001$). There were however, no significant differences ($p>0.05$) in the killing activity of NK cell between the mice

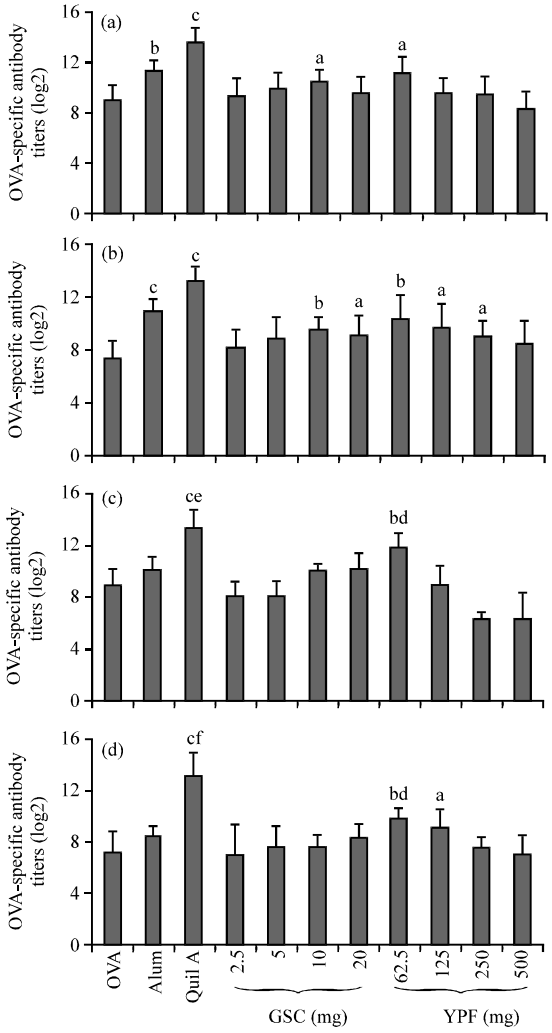

Fig. 3: Effect of oral administration of GSC and YPF on OVA-specific IgG, IgG1, IgG2a and IgG2b antibody in OVA-immunized mice. The values are presented as mean±SD (n = 6). Significant differences with OVA control group were designated as $^{a}p<0.05$, $^{b}p<0.01$ and $^{c}p<0.001$; those with OVA/Alum group as $^{d}p<0.05$, $^{e}p<0.01$ and $^{f}p<0.001$; a) IgG; b) IgG1; c) IgG2a and d) IgG2b

groups immunized with OVA/Alum and OVA alone. The findings indicated that GSC and YPF could promote NK cell lytic activities in the OVA-immunized mice.

Effect of oral administration of GSC and YPF on the specific serum antibody response: The OVA-specific IgG, IgG1, IgG2a and IgG2b antibody levels in the serum were measured 2 weeks after the last immunization using ELISA and the results were shown in Fig. 3. Alum, Quil A, GSC and YPF could significantly enhance the OVA-specific

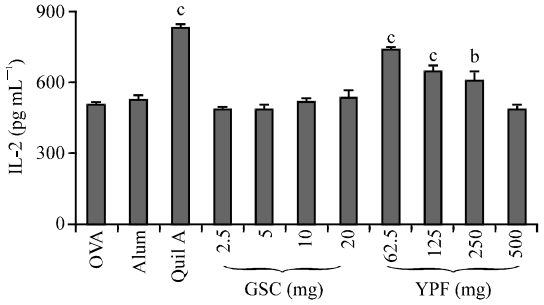

Fig. 4: Effect of oral administration of GSC and YPF on cytokine production from splenocytes in the OVA-immunized mice. The values are presented as mean±SD (n = 6). Significant differences with OVA alone and OVA/Alum groups were designated as $^{b}p<0.01$ and $^{c}p<0.001$

IgG and IgG1 titers in the immunized mice ($p<0.05$, $p<0.01$ or $p<0.001$). Significant enhancements in OVA-specific serum IgG2a and IgG2b titers were also observed in Quil A and YPF-immunized mice compared with OVA alone group ($p<0.05$, $p<0.01$ or $p<0.001$). Moreover, IgG2a and IgG 2b antibody titers in the mice immunized with YPF at the dose of 62.5 mg (equivalent to crude drug) were higher than those in the Alum-treated mice ($p<0.05$). There were however, no significant differences ($p>0.05$) in the serum IgG2a and IgG2b levels among mice groups immunized with OVA/GSC, OVA/Alum and OVA alone. Collectively, the findings indicated that GSC and YPF significantly enhanced serum OVA-specific antibody production in mice immunized with OVA. Moreover, the type of antibody response induced by YPF-adjuvanted OVA after two injections were better than that elicited by Alum-adjuvanted OVA.

Effect of oral administration of GSC and YPF on cytokine secretion from splenocytes: In order to verify the ability of YPF to induce Th1 immune response to OVA, the production of Th1 cytokine IL-2 from splenocytes in OVA-immunized mice was detected using ELISA. The calibration curve of IL-2 was constructed with mouse cytokine standards and its correlation coefficients were all bigger than 0.9980. As shown in Fig. 4, the contents of cytokines IL-2 in the supernatants from cultured splenocytes in the mice immunized with OVA/YPF and OVA/Quil A were significantly higher than those in OVA control mice ($p<0.01$ or $p<0.001$), suggesting that YPF significantly enhanced the production of the Th1 cytokines in OVA-immunized mice. However, there were no significant differences ($p>0.05$) in IL-2 levels among mice groups immunized with OVA/GSC, OVA/Alum and OVA alone.

Table 1: Effect of oral administration of GSC and YPF on the body weight of mice

Groups	Weight (g)[a]	
	Before treatment	After treatment
Saline	24.50±0.79	30.83±1.53
OVA	24.53±0.52	31.50±3.57
Alum	24.83±1.22	31.75±2.31
Quil A	24.73±1.35	31.42±3.03
GSC (2.5 mg)[b]	24.90±1.10	34.18±3.32
GSC (5.0 mg)	24.70±1.10	34.07±2.60
GSC (10 mg)	24.70±1.44	32.82±3.23
GSC (20 mg)	24.87±1.28	33.50±2.62
YPF (62.5 mg)[b]	24.77±1.40	32.77±1.83
YPF (125 mg)	24.68±1.08	33.93±3.13
YPF (250 mg)	24.75±1.20	33.10±2.12
YPF (500 mg)	24.67±1.27	33.27±2.84

[a]The values are presented as means±SD (n = 6); [b]The doses of GCS and YPF were expressed by preparation and crude drug per mouse daily, respectively

Effect of oral administration of GSC and YPF on the body weight of mice: As shown in Table 1, the treatment of mice with GSC or YPF did not produce any significant changes in body weights compared to saline-treated mice during the experimental period. In addition, no signs of toxicity were observed in the GSC and YPF-treated mice on the basis of microscopic examination of individual organs (intestinal tract, liver and kidney). These results suggest that GSC and YPF treatment was well-tolerated during the experimental period in this study.

DISCUSSION

An ideal adjuvant should promote an appropriate immune response, be stable with long shelf life, biodegradable, cheap to produce and not themselves immunogenic (Tritto *et al*., 2009). While several hundred different adjuvants have been proposed over the last few decades, the vast majority have not been successful in being approved for human use with limitations including lack of efficacy, unacceptable local or systemic toxicity, manufacturing difficulties, instability and prohibitive cost (Aguilar and Rodriguez, 2007). Therefore, there is an increasing need to develop novel vaccine adjuvants able to augment antigen-specific immunity. This is particularly true for vaccines containing antigens that are either highly purified, need to be active at the mucosal surface or required certain kinds of protective immune responses to be induced depending on the disease context.

Many traditional Chinese medicines are said to possess a tonic effect that assists the body in the maintenance of health and could mediate such effects through targeted modulation of antigen-presenting cells, T and B-lymphocytes (Tritto *et al*., 2009). Although, there are several hundred publications on the apparent immune-enhancing effects of traditional Chinese medicines many of the reports are based on *in vitro* studies. There is no doubt that their extracts can stimulate the immune system when injected systemically or *in vitro*. However, the traditional Chinese medicines are usually administered orally in the form of formulas and systemic injections are very rarely applied. The oral use of the traditional Chinese medicines as adjuvants can avoid the side effects found in parenteral administration. In the context of vaccination, developing countries with low vaccination coverage and increased risk of disease associated with injectable vaccines could greatly benefit from the use of plant-derived medicines as mucosal adjuvants which may prove to be a simple, cost-effective and potentially life-saving health care approach. Therefore, the studies describing immune-enhancing effects of traditional Chinese medicines following oral administration has become an increasingly important area of research (Tritto *et al*., 2009). The current study was undertaken to evaluate the adjuvant potential of two Chinese medicine prescription preparations GSC and YPF following oral administration on the cellular and humoral immune responses of mice against OVA.

The cellular immune response plays an important role in the host response to intracellular pathogens by limiting replication and accelerating clearance of infected cells and in the generation of both humoral and cell-mediated responses to vaccination. The capacity to elicit an effective T and B-lymphocyte immunity can be shown by the stimulation of lymphocyte proliferation response (Marciani *et al*., 2000). It is generally known that Con A stimulates T cells and LPS stimulates B cell proliferation. The proliferation assay showed that GSC and YPF could significantly promote the Con A, LPS and OVA-stimulated splenocyte proliferation in the immunized mice. The results indicated that GSC and YPF could significantly increase the activation potential of T and B cells and induce the humoral immunity and cell-mediated immune response in the OVA-immunized mice.

The NK cell-activity assay is also a routine method for the analysis of the cellular immune response *in vitro* (Orange, 2008). In this investigation, GSC and YPF significantly enhanced the lytic activity of NK cells in OVA-immunized mice, suggesting that YPF could improve cytolytic activities against autologous tumor cells and viruses.

Evidence now exists to clearly suggest that Th1 or Th2 responses, generated upon antigenic stimulation can be modulated *in vivo* depending on the adjuvant used for immunization (Audibert and Lise, 1993). The Th1 immune response is characterized by the production of cytokines IL-2, TNF-β and IFN-γ and an enhanced production of IgG2a, IgG2b and IgG3 in mice, correlated with the

induction of cell-mediated immunity is required for protective immunity against intracellular infectious agents such as viruses, certain bacteria and protozoa and presumably against cancer cells (Mosmann and Sad, 1996). The Th2 response is characterized by the production of cytokines IL-4, IL-5 and IL-10 and an enhanced production of IgG1 and secretary IgA (Livingston *et al*., 1994). Th2 immunity which controls the humoral immune response through the triggering of B cell proliferation and differentiation is effective for protection against most bacterial as well as certain viral infections (McKee *et al*., 2007). In this study, the adjuvant activity of GSC and YPF on the humoral immune responses to OVA was also evaluated. YPF significantly not only enhanced OVA-specific IgG and IgG1 titers but increased specific IgG2a and IgG2b antibody titers in immunized mice. However, Alum and GSC only promoted the serum specific IgG and IgG1 antibody levels in the OVA-immunized mice. The cytokine assay also showed that YPF significantly increased the production of IL-2 from splenocytes in the OVA-immunized mice. This clearly demonstrated that in addition to enhancing the magnitude of antibody responses, YPF also modulated the quality of immune responses and elicited a balanced Th1/Th2 immune response to OVA in mice as associated sensitively with an enhancement of IgG2a, IgG2b and IgG1 levels (Finkelman *et al*., 1990) and that the adjuvant property of YPF on antibody immune responses were superior to those of Alum.

GSC is comprised of the extracts from *Echinacea purpurea* and *Astragalus membranaceus*. *Echinacea* sp. commonly known as purple cone flower is a top-selling herbal remedy that purportedly acts as an immunostimulant. Today, this medicinal plant is used clinically to reduce the symptoms of the common cold and of upper respiratory tract infections (Schoop *et al*., 2006). Although, *Echinacea* sp. is still widely claimed to be an immunostimulant both in the popular and scientific literature (Yin *et al*., 2010) this is remarkable as no sound data exist to show irrefutably that purple cone flower extracts activate the immune system when administered orally. In more recent years, it has become apparent that the purple cone flower contains potent bioavailable anti-inflammatory principles which inhibit TNF-α and IL-1β expression and induce a Th1/Th2 cytokine shift and that it can be immunostimulatory, immunosuppressive and/or anti-inflammatory depending on the portion of the plant and extraction method (Benson *et al*., 2010). Recently, Ragupathi *et al*. (2008) evaluated the adjuvant activities of three different forms of Echinacea: lipophilic extract of *E. angustifolia* and *E. purpurea* roots, neutral and weak acidic polysaccharides from *E. purpurea* juice extract and strong acidic polysaccharides from *E. purpurea* juice extract as well as three extracts of *A. membranaceus* prepared using water, 50% ethanol and 95% ethanol as solvents. The results showed that 95% ethanol extract of Astragalus had potent adjuvant activity but Echinacea extracts or the Astragalus water extract had little or no adjuvant activity. The components responsible for this adjuvant activity in 95% ethanol extract of Astragalus were also further identified and found to be astragalosides II, IV and several flavonoids. However, when the activities of these known immunologically active components of Astragalus are calculated based on the extent of their presence in the 95% ethanol extract they provide only a small proportion of the immunological activity (Hong *et al*., 2011). They raised the possibility that additional uniquely active components of Astragalus may contribute to adjuvant activity or that the adjuvant activity of Astragalus is greater than the activity of the sum of its parts. These results may account, at least in part for the fact that little adjuvant activity was demonstrated with GSC in the study.

In the study, the oral administration of GSC and YPF did not affect body weight of mice. No abnormal behavior and side effects were yet observed in mice throughout the experiment. The observation suggests that these two preparations were well-tolerated by the mice.

CONCLUSION

YPF was better than GSC and alum in the quality and strengths to enhance specific cellular and humoral immune responses against OVA in mice and could simultaneously elicit a Th1 and Th2 immune responses. Taken together with its natural origin without lethal toxicity to humans and animals and long-standing use as traditional Chinese medicines, YPF may be a safe and efficacious mucosal adjuvant candidate suitable for a wide spectrum of vaccines for which a balanced and potent stimulation of both the cellular and humoral responses is required. The studies on YPF with the various types of antigen including vaccine clinically used in other animal models are in progress to verify the adjuvant effect.

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