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Novel polysaccharide adjuvant from the roots of *Actinidia eriantha* with dual Th1 and Th2 potentiating activity

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ABSTRACT

The plant polysaccharides are recognized as an effective biological response modifier with low toxicity. In this study, the water-soluble polysaccharide from the roots of *Actinidia eriantha* (AEPS) was evaluated for its toxicity and adjuvant potential on the specific cellular and humoral immune responses to ovalbumin (OVA) in mice. AEP did not cause any mortality and side effects when mice were administered subcutaneously twice at the dose up to 5.0 mg at intervals of 7 days. The mice were immunized subcutaneously with OVA 100 μg alone or with OVA 100 μg dissolved in saline containing Quil A (10 μg) or AEPS (25, 50, or 100 μg) on days 1 and 15. Two weeks later, splenocyte proliferation, natural killer (NK) cell activity, production and mRNA expression of cytokines from splenocytes, and serum OVA-specific antibody titers were measured. The Con A-, LPS-, and OVA-induced splenocyte proliferation and the serum OVA-specific IgG, IgG1, IgG2a, and IgG2b antibody titers in the immunized mice were significantly enhanced by AEPS ($P<0.05$, $P<0.01$ or $P<0.001$). AEPS also significantly promoted the production of Th1 (IL-2 and IFN-γ) and Th2 (IL-10) cytokines and up-regulated the mRNA expression of IL-2, IFN-γ, IL-4 and IL-10 cytokines and T-bet and GATA-3 transcription factors in splenocytes from the immunized mice ($P<0.05$, $P<0.01$ or $P<0.001$). Besides, AEPS remarkably increased the killing activities of NK cells from splenocytes in the immunized mice ($P<0.01$ or $P<0.001$). The results indicated that AEPS had strong potential to increase both cellular and humoral immune responses and elicit a balanced Th1/Th2 response, and that AEPS may be a safe and efficacious adjuvant candidate suitable for a wide spectrum of prophylactic and therapeutic vaccines.

1. Introduction

Vaccination remains the most cost-effective biomedical approach for the control of infectious diseases. The safety of the traditional vaccines based on live attenuated or killed microorganisms is questionable due to the risk of virulence reversion [1]. New generations of vaccines based on purified recombinant proteins, synthetic peptides and plasmid DNA, despite their better tolerability, are unfortunately often much less reactogenic and immunogenic [2]. The majority of these vaccines require association with adjuvants capable of increasing the potency or stimulating the appropriate immune response [3,4]. The benefits flowing from adjuvant incorporation into any vaccine formulation have to be balanced with the risk of adverse reactions induced by these compounds. Unfortunately, strong adjuvant activity is often correlated with increased toxicity. Freund's complete adjuvant (FCA) remains amongst the most potent of known adjuvants and a particularly powerful stimulant of both cellular and humoral immunities [5]. Unfortunately FCA causes severe reactions and is too toxic for human use. The unique capacity of the extract Quil A from the bark of *Quillaja saponaria* and its purified saponin QS-21 to stimulate both the Th1 immune response and the production of cytotoxic T-lymphocyte against exogenous antigens makes them ideal for use in subunit vaccines and vaccines directed against intracellular pathogens as well as for therapeutic cancer vaccines [6,7]. However, in addition to pain on injection, severe local reactions and granulomas, toxicity includes severe haemolysis [8–11] making such adjuvants unsuitable for human uses other than for life threatening diseases, such as HIV infection or cancer [12]. Although muramyl dipeptide (MDP) and other derivatives from Gram-negative bacteria, such as lipopolysaccharide (LPS) and monophosphoryl lipid A have also been used as human adjuvants, their toxicities remain the single biggest barrier to the use of such adjuvants for human prophylactic vaccines [13,14]. A major challenge in adjuvant research is to increase adjuvant activity while reducing toxicity [8]. Currently, aluminum compounds

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(Alum) are the only vaccine adjuvants licensed by the Food and Drug Administration (FDA) for use in humans [15]. While Alum is safe, it is a relatively weak adjuvant particularly when used with subunit antigens. Moreover, the Alum is a mild Th2 adjuvant that can effectively enhance IgG1 antibody responses, but it is rarely associated with Th1 type immune responses [16]. Furthermore, Alum is poor at stimulating cell-mediated immune responses, and may actively block activation and differentiation of cytotoxic T-lymphocytes [17]. Hence, there is a major unmet need for a safe and efficacious adjuvant capable of boosting cellular plus humoral immunity [18].

Most polysaccharides derived from higher plants are relatively nontoxic and do not cause significant side effects, which is a major problem associated with immunomodulatory bacterial polysaccharides and synthetic compounds. Thus, plant polysaccharides are recognized as an effective biological response modifier with low toxicity [19]. Recently, many polysaccharides have been shown to possess adjuvant potential on specific cellular and humoral immune responses against antigen and vaccine and be excellent candidates to replace Alum as the adjuvant of choice for many vaccines [20–29]. Particular advantages offered by plant polysaccharide adjuvants in inducing cellular in addition to humoral immunity offer excellent safety, tolerability, ease of manufacture and formulation. Thus, the plant polysaccharide adjuvants have enormous potential for use in vaccines against both pathogens and cancer [18].

The genus *Actinidia* (Actinidiaceae) consists of over 58 species and widely distributed in the Asian continent. Most species are native to temperate regions of south-western China. *Actinidia* fruits are nutritious fruits distinguishable from other fruits by the attractive green color of their flesh [30]. Some *Actinidia species*, such as *Actinidia macrosperma*, are the important traditional medicine [31]. The roots of *A. eriantha* Benth have been used for gastric carcinoma, nasopharyngeal carcinoma, breast carcinoma, and hepatitis in traditional Chinese medicine [32]. The modern pharmacological experiments also proved that the water extracts of this drug possessed antitumor and immunopotentiating activities [33]. We previously reported that the water-soluble polysaccharide (AEPS) was the main active principles of the antitumor and immunomodulatory effect of this drug [34]. Ovalbumin (OVA) is commonly used as the model antigen to detect the adjuvant effect of chemicals [35]. We have previously used OVA as the model antigen to screen the saponins with the adjuvant properties from Chinese traditional medicines [36]. The obtained saponins with the adjuvant properties have also been verified their adjuvant effects on recombinant hepatitis B surface vaccine, Newcastle disease virus-based recombinant avian influenza vaccine, and recombinant fowlpox virus vector-based avian influenza vaccine in the mice and chickens [37,38]. In this study, in order to investigate the immunoadjuvant property of the isolated polysaccharide AEPS, its toxicity and adjuvant potential on the cellular and humoral immune responses to OVA in mice were investigated.

2. Materials and methods

2.1. Mice

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 the Institute for Experimental Animals of Zhejiang University and were approved by the University Committee for animal experiments.

2.2. Materials

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., Saint Louis, MO, USA; goat anti-mouse IgG1, IgG2a and IgG2b peroxidase conjugate were from Southern Biotech. Assoc., Birmingham, AL, USA; cytokine (IL-2, IL-10, and IFN-γ) detecting ELISA kits were from Wuhan Boster Biological Technology Co. Ltd., Hubei, China. Quil A was kindly provided by Brenntag Nordic A/S, Denmark. Trizol was from Invitrogen, USA; revert Aid^{TM} M-MuLV reverse transcriptase was from Fermentas, USA; diethylpyrocarbonate (DEPC) and ribonuclease inhibitor were from Biobasic, Canada; oligo(dT)$_{18}$ were from Sangon, China. 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 (Sigma), 100 IU/ml penicillin, 100 μg/ml streptomycin (Sigma), and 10% fetal calf serum at 37 °C under humidified air with 5% CO_2.

2.3. Extraction, purification and characterization of polysaccharide AEPS

The roots of *A. eriantha* were collected in Wuyi county, Zhejiang province, China in August 2007. A voucher specimen (No. 20070806) has been deposited at the Laboratory of Nature Drug, College of Animal Sciences, Zhejiang University, China. The plant material (1 kg) was extracted with boiling water three times under reflux. The aqueous extract was filtered through filter paper. The filtrate was concentrated under reduced pressure, and then centrifuged at 3000 rpm for 15 min. The supernatant was precipitated with three volumes of 95% ethanol, and stored overnight at 4 °C. The resulting precipitate (49.4 g) was dissolved in distilled water and dialyzed against distilled water (cut-off M_w 7000 Da). The retentate portion was subjected to DEAE–Sephadex A-50 column chromatography, washed with H_2O, and eluted with 1.0 M sodium chloride solution. The eluates collected were concentrated, dialyzed and lyophilized to afford a total *A. eriantha* polysaccharide (AEPS, light off-white powder, 34.7 g).

Total sugar content was estimated by the phenol–sulfuric acid analysis using glucose as standard [39]. Uronic acid content was determined by the carbazole–sulfuric acid method using glucuronic acid as standard [40]. Neutral monosaccharide composition was analyzed according to the following procedure: AEPS (5 mg) were hydrolyzed with 5 ml of 2 M TFA at 110 °C for 5 h to release component monosaccharides. The hydrolyzed monosaccharides (inositol as the internal standard) were derivatized to acetylated aldononitriles [41] and isothermally separated by gas chromatography (GC) in an Agilent 4890D system (Agilent Technologies, Palo Alto, CA, USA) equipped with a flame-ionization detector (FID) and a DB-35 capillary column (30.0 m × 0.32 nm × 0.25 μm) [34].

A stock AEPS solution with a concentration of 10 mg/ml was prepared by dissolving in 0.89% saline. The solution was sterilized by passing it through a 0.22-μm Millipore filter, and then analyzed for endotoxin level by a gel-clot *Limulus* amebocyte lysate assay (Zhejiang A and C Biological, Zhejiang, China).

2.4. Toxicity assays

Six-week-old female ICR mice were divided into five groups, each consisting of five mice. Animals were injected twice subcutaneously on the back with AEPS at a single dose of 0.5, 1.0, 2.5, 5.0 mg in 0.5 ml saline solution at weekly intervals, and monitored daily for 14 days. Saline-treated animals were included as control group and the toxicity was assessed by lethality, local swelling and loss of hair at the site of injection.

2.5. Immunization

Six-week-old female ICR mice were divided into six groups, each consisting of five mice. Animals were immunized subcutaneously with OVA 100 μg alone or with OVA 100 μg dissolved in saline containing Quil A (10 μg) or AEPS (25, 50, or 100 μg) on day 1. Saline-treated animals were included as 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.

2.6. Splenocyte proliferation assay

Spleen collected from the OVA-immunized mice under aseptic conditions, in Hank's balanced salt solution (HBSS, Sigma), was minced using a pair of scissors and passed through a fine steel mesh to obtain a homogeneous cell suspension, and the erythrocytes were lysed with ammonium chloride (0.8%, w/v). After centrifugation (380 × *g* at 4 °C for 10 min), the pelleted cells were washed three times in PBS, and resuspended in complete medium. Cell numbers were counted with a haemocytometer by trypan blue dye exclusion technique. Cell viability exceeded 95%. Splenocyte proliferation was assayed as previously described [42]. Briefly, splenocytes were seeded into four wells of a 96-well flat-bottom microtiter plate at 5×10^6 cell/ml in 100 μl complete medium, thereafter Con A (final concentration 5 μg/ml), LPS (final concentration 10 μg/ml), OVA (final concentration 20 μg/ml), 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) 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 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 with a 630 nm reference after 15 min. The stimulation index (SI) was calculated based on the following formula: SI = the absorbance value for mitogen-cultures divided by the absorbance value for non-stimulated cultures.

2.7. Measurement of OVA-specific antibody

OVA-specific IgG, IgG1, IgG2a, and IgG2b antibodies in sera were detected by an indirect ELISA. In brief, microtiter plate wells (Nunc) were coated with 100 μl OVA solution (25 μg/ml 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 1:8000, 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 of 2N 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.

2.8. Assay of natural killer (NK) cell activity

The activity of NK cells was measured as previously described [43]. Briefly, K562 cells were used as target cells and seeded in 96-well U-bottom microtiter plate (Costar) 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. 50 μl of MTT solution (2 mg/ml) 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: NK activity (%) = $(OD_T - (OD_S - OD_E))/OD_T \times 100$, where OD_T, optical density value of target cells control, OD_S, optical density value of test samples and OD_E, optical density value of effector cells control.

2.9. Cytokine measurements in the cultured supernatants of splenocytes by ELISA

Splenocytes (5×10^5 cells/well) from the immunized mice prepared as described before were incubated with Con A (final concentration 5 μg/ml) 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, IL-10, and IFN-γ levels using commercial ELISA kits. Briefly, culture supernatants or cytokine standards were added to 96-well flat-bottom microtiter plates coated with coating antibody, and plates then incubated at 37 °C for 1.5 h (IL-2 and IL-10) or 2 h (IFN-γ). 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.

2.10. RT-PCR for cytokine and transcription factor gene expression

Splenocytes from the immunized mice prepared as described before were seeded into a 24-well flat-bottom microtiter plate at 5 × 106 cell/ml in 1 ml complete medium, thereafter Con A (final concentration 5 μg/ml) was added giving a final volume of 2 ml (triplicate wells). The plates were incubated at 37 °C in a humidified atmosphere with 5% CO_2. After 12 h treatment, cells were harvested by centrifugation (380 × *g* at 4 °C for 10 min), and washed with ice-cold PBS, then subjected to RNA extraction. Splenocytes were lysed in 0.8 ml of Trizol reagent and the total RNA was isolated according to the manufacturer's protocol. The concentration of total RNA was quantified by determining the optical density at 260 nm. The total RNA was used and reverse transcription was performed by mixing 2 μg of RNA with 0.5 μg oligo$(dT)_{18}$ primer in a sterile tube. Nuclease-free water was added giving a final volume of 12.5 μl. This mix was incubated at 70 °C for 5 min and chilled on ice for 2 min. Then a solution containing 4 μl of M-MuLV 5× reaction buffer, 2 μl of 10 mM dNTP, 20 U of ribonuclease inhibitor, and DEPC-treated water was added giving a final volume of 19 μl and the tubes were

Table 1
Sequences of primer used for RT-PCR.

Gene	Primer sequence	Product size (bp)
GAPDH	5′-CCCACAGTAAATTCAACGGCAC-3′ 5′-CATTGGGGTTAGGAACACGGA-3′	570
IL-2	5′-CTCTACAGCGGAAGCACAGC-3′ 5′-CATCTCCTCAGAAAGTCCACCA-3′	381
IFN-γ	5′-TGAACGCTACACACTGCATCTTGG-3′ 5′-CGACTCCTTTTCCGCTTCCTGAG-3′	459
IL-4	5′-ATGGGTCTCAACCCCCAGCTAGT-3′ 5′-GCTCTTTAGGCTTTCCAGGAAGTC-3′	399
IL-10	5′-CCAGTTTTACCTGGTAGAAGTGATG-3′ 5′-TGTCTAGGTCCTGGAGTCCAGCAGACTCAA-3′	324
T-bet	5′-AACCAGTATCCTGTTCCCAGC-3′ 5′-TGTCGCCACTGGAAGGATAG-3′	436
GATA-3	5′-GAAGGCATCCAGACCCGAAAC-3′ 5′-ACCCATGGCGGTGACCATGC-3′	255

GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

incubated for 5 min at 37 °C. The tubes then received 200 U of M-MuLV reverse transcriptase and were incubated for 60 min at 42 °C. Finally, the reaction was stopped by heating at 70 °C for 10 min. The samples were stored at − 20 °C until further use.

Amplification was carried out in a total volume of 20 μl containing 0.5 μl (10 μM) of each cytokine- or transcription factor-specific primers (Table 1), 2 μl of 10× PCR buffer, 1.2 μl of $MgCl_2$ (25 mmol/l), 0.5 μl of dNTP (10 mM), 1 μl of transcribed cDNA, and 0.25 μl of Taq DNA polymerase. PCR was performed for 27 (GAPDH and IFN-γ), 29 (GATA-3), 31 (IL-2 and T-bet), or 33 (IL-4 and IL-10) cycles using a PTC-200 Thermal Cycler (MJ Research, Watertown, MA) with the following program of denaturation at 94 °C for 1 min, annealing at 55 °C (IL-2, GATA-3, and T-bet), 57 °C (GAPDH), or 58 °C (IL-4, IL-10, and IFN-γ) for 50 s, and elongation at 72 °C for 0.5 min. Semi-quantitative RT-PCR was performed using GAPDH as an internal control to normalize gene expression for the PCR templates. The PCR products were studied on a 1.5% agarose gel and the amplified bands were visualized using ImmageMaster VDS Software (Phamacia Biotech, USA) after staining with GoldView. The size of the amplified fragments was determined by comparison with a standard DNA marker.

2.11. Statistical analysis

The data were expressed as mean ± standard errors (S.E.) and examined for their statistical significance of difference with ANOVA and a Tukey post hoc test. *P*-values of less than 0.05 were considered to be statistically significant.

3. Results

3.1. Isolation, characterization and composition of AEPS

The crude polysaccharide was extracted with hot water from the roots of *A. eriantha* and purified by means of DEAE–Sephadex A-50 gel chromatography, leading to a water-soluble polysaccharide AEPS. AEPS showed positive sulfuric acid–carbazole reaction, suggesting that it contained uronic acid and negative Fehling's reagent and iodine–potassium iodide reactions, indicating that it did not contain reducing sugar and starch-type polysaccharides. UV analysis and triketohydrindene hydrate reaction indicated that AEPS was not contaminated with proteins. GC quantitative analysis with derivatization revealed that AEPS was composed of rhamnose (3.18 mol.%), fucose (14.85 mol.%), arabinose (26.87 mol.%), xylose (4.49 mol.%), mannose (7.83 mol.%), glucose (7.72 mol.%), and galactose (35.05 mol.%) with the molar ratio of 1.00:4.67:8.45:1.41:2.46:2.43:11.02. The uronic acid content in AEPS was 3.70%.

3.2. Toxicity of AEPS

The endotoxin level in a stock AEPS solution with a concentration of 10 mg/ml was measured to be less than 0.5 endotoxin units (EU)/ml. Therefore, the AEPS sample used in this study was excluded from endotoxin contamination. When the animals were administered subcutaneously twice ranging from 0.5 to 5.0 mg at weekly intervals, there is no lethality observed. Local swelling or loss of hair was not observed in mice at the tested doses. The results suggested that the safety dose of AEPS used for human and animal was at least up to 200 mg/kg.

3.3. Effect of AEPS on splenocyte proliferation in OVA-immunized mice

The effects of AEPS on mitogen- and OVA-stimulated splenocyte proliferation in the immunized mice are shown in Fig. 1. As a positive control, Quil A markedly enhanced Con A-, LPS-, and OVA-stimulated splenocyte proliferation in the immunized mice. AEPS also significantly increased Con A- and LPS-stimulated splenocyte proliferation in the OVA-immunized mice ($P < 0.05$ or $P < 0.01$). The OVA-induced splenocyte proliferation in the immunized mice was also similarly increased by AEPS at three doses compared to OVA group ($P < 0.01$ or $P < 0.001$).

3.4. Effect of AEPS on the OVA-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. 2. OVA

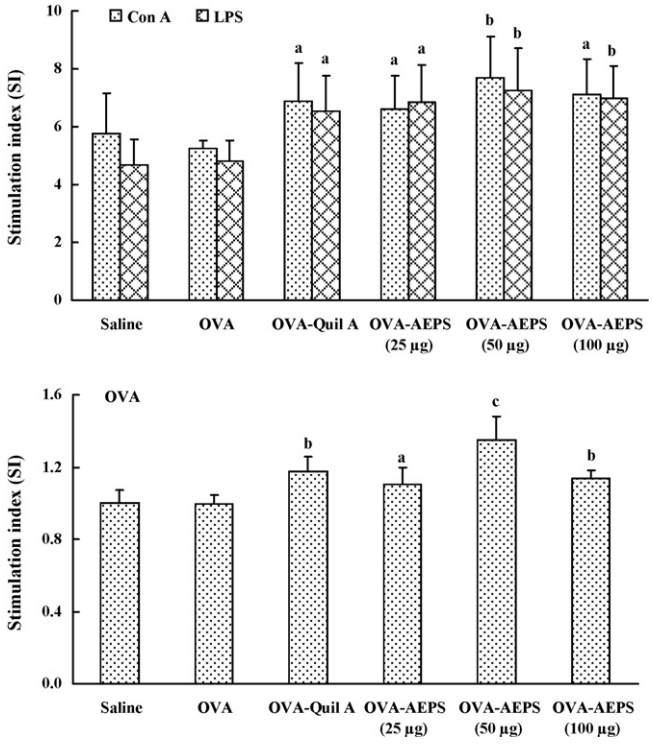

Fig. 1. Effect of AEPS on mitogen- and OVA-stimulated splenocyte proliferation in the mice immunized with OVA. Significant differences with OVA alone group were designated as $^{a}P < 0.05$, $^{b}P < 0.01$, and $^{c}P < 0.001$.

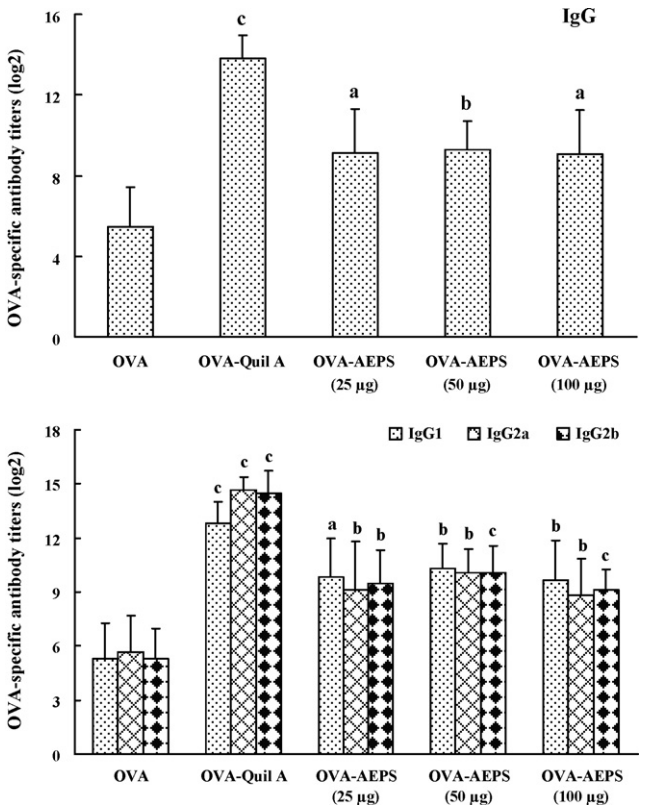

Fig. 2. Effect of AEPS on OVA-specific IgG, IgG1, IgG2a, and IgG2b antibodies in OVA-immunized mice. Significant differences with OVA alone groups were designated as $^{a}P<0.05$, $^{b}P<0.01$, and $^{c}P<0.001$.

alone induced low levels of OVA-specific IgG antibody. However, addition of Quil A and AEPS to OVA resulted in a significant increase in total IgG antibody titers ($P<0.05$, $P<0.01$, or $P<0.001$). The OVA-specific serum IgG1 titers in OVA-immunized mice were also significantly increased by Quil A and AEPS ($P<0.05$, $P<0.01$, or $P<0.001$). With regard to OVA-specific serum IgG2a and IgG2b titers, mice immunized with OVA/Quil A and OVA/AEPS had a dramatic increase compared with OVA alone group ($P<0.01$ or $P<0.001$). The findings indicated that AEPS significantly enhanced serum OVA-specific antibody production in immunized mice.

3.5. Effects of AEPS on NK cell activity in OVA-immunized mice

The effects of AEPS on NK cell activity in OVA-immunized mice were shown in Fig. 3. AEPS and Quil A significantly enhanced the killing activity of NK cell in the OVA-immunized mice ($P<0.01$ or $P<0.001$). The findings indicated that AEPS could promote lytic activity of NK cells in mice immunized with OVA.

3.6. Effect of AEPS on cytokine secretion by splenocytes from OVA-immunized mice

The calibration curves of IL-2, IFN-γ and IL-10 were constructed with mouse cytokine standards, and their correlation coefficients were all bigger than 0.9980. As shown in Fig. 4, the contents of cytokines IL-2, IFN-γ, and IL-10 in the supernatants from cultured splenocytes in the mice immunized with OVA/AEPS and OVA/Quil A were significantly higher than those in OVA control mice ($P<0.01$ or $P<0.001$), suggesting that AEPS significantly enhanced the production of the Th1 and Th2 cytokines in OVA-immunized mice.

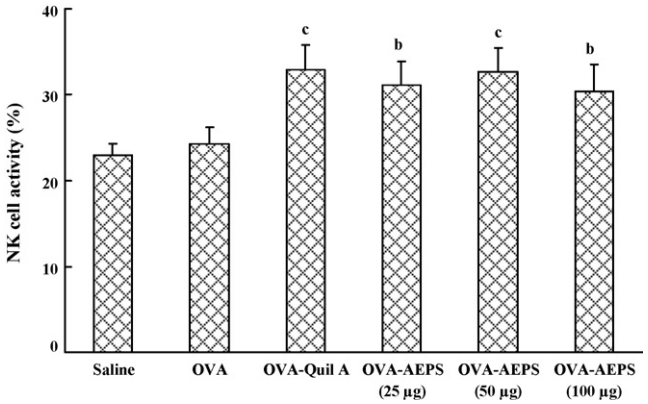

Fig. 3. Effect of AEPS on NK cell activity in mice immunized with OVA. Significant differences with OVA alone group were designated as $^{b}P<0.01$ and $^{c}P<0.001$.

3.7. Effect of AEPS on mRNA expression of cytokines and transcription factors in splenocytes from the immunized mice

As shown in Fig. 5 and Table 2, AEPS and Quil A significantly not only increased the mRNA expression of Th2 cytokines IL-4 and IL-10 and transcription factor GATA-3 ($P<0.01$ or $P<0.001$), but enhanced that of Th1 cytokines IL-2 and IFN-γ and transcription factor T-bet ($P<0.05$, $P<0.01$, or $P<0.001$) in splenocytes from the immunized mice. Thus, findings showed that AEPS up-regulated the gene expression of Th1/Th2 cytokines and transcription factors in splenocytes from the immunized mice.

4. Discussion

Despite technological advances in molecular biology and in genetic engineering, allowing identification of antigens with

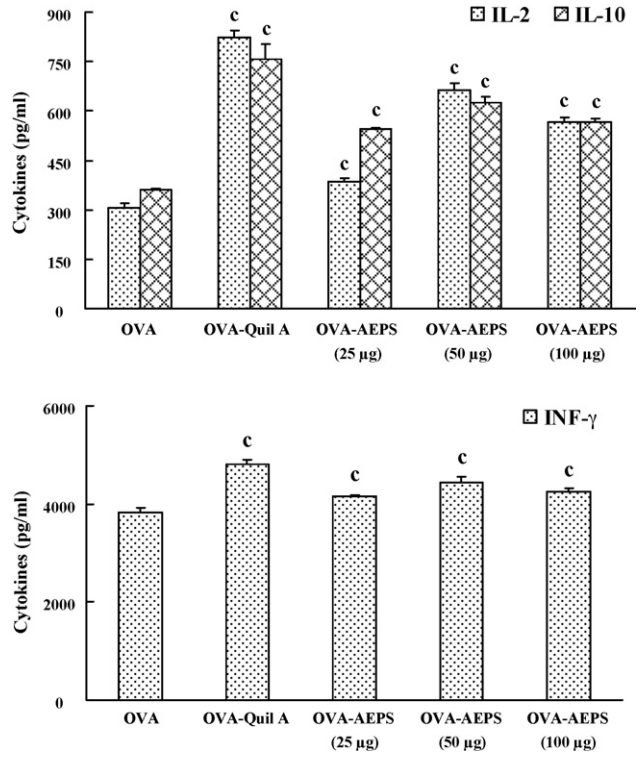

Fig. 4. Effects of AEPS on cytokine production in splenocytes from the OVA-immunized mice. Significant differences with OVA alone group were designated as $^{c}P<0.001$.

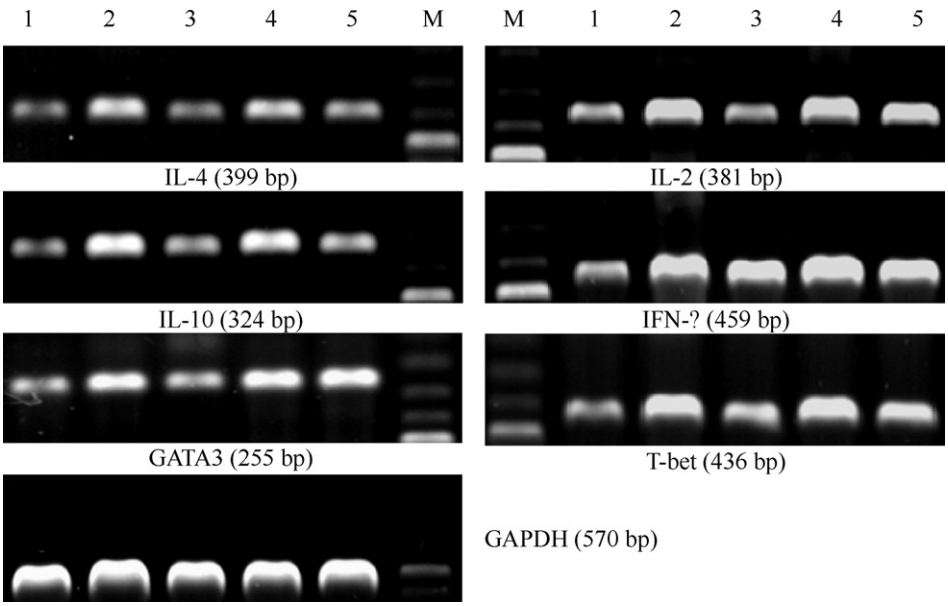

Fig. 5. Effect of AEPS on the mRNA expression of GAPDH, cytokines and transcription factors in splenocytes from the OVA-immunized mice. Lane M, DNA marker; lane 1, OVA; lane 2, OVA–Quil A; lane 3, OVA–AEPS (25 μg); lane 4, OVA–AEPS (50 μg); lane 5, OVA–AEPS (100 μg).

immunogenic potential, the majority of vaccines requires association with adjuvants capable of increasing the potency or stimulating the appropriate immune response [4,44]. An efficient adjuvant should have negligible toxicity and enhances the humoral or/and cellular immune response to a specific antigen [45]. Ideally, adjuvants should promote an appropriate immune response, be stable with long shelf life, biodegradable, cheap to produce and not themselves immunogenic [46]. 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 [47].

In our previous work, the saponins from the root of *Achyranthes bidentata* [48], *Astragalus membranaceus* [49], *Bupleurum chinense* [50], *Glycyrrhiza uralensis* [51], *Panax notoginseng* [42], *Platycodon grandiflorum* [52] and the herb of *Gynostemma pentaphyllum* [53] were shown to possess the adjuvant potentials on the cellular and humoral immune response of mice against OVA. Although the haemolytic activities and toxicities of these saponins were lower than that of Quil A, most saponins still had some haemolytic effects. In addition, the saponins have a strong adjuvant activity when administered parenterally, in general, while they have a low or no activity when delivered orally [36]. The extensive studies on plant polysaccharide adjuvants indicate that these are excellent candidates to replace Alum as the adjuvant of choice for many vaccines. Meanwhile, most polysaccharides could exert the similar pharmacological effects when administered parenterally and orally. AEPS have been proved to significantly increase splenocytes proliferation, NK cell and CTL activity, IL-2 production from splenocytes, and serum antigen-specific antibody levels in S180 sarcoma-bearing mice by oral administration route [34]. To further search the safer adjuvant, the current study was undertaken to evaluate the toxicity of AEPS and its adjuvant potential 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. Among the T-lymphocytes, helper T cells induce B-lymphocytes to secrete antibodies, and cytotoxic T-lymphocytes help phagocytes to destroy ingested microbes and to kill intracellular microbes. Humoral immunity, however, mediated by antibodies, which are produced by B-lymphocytes, functions by neutralizing and eliminating extracellular microbes and microbial toxins. The capacity to elicit an effective T- and B-lymphocyte immunity can be shown by the stimulation of lymphocyte proliferation response. It is generally known that Con A stimulates T cells and LPS stimulates B cell proliferation. The proliferation assay showed that AEPS could significantly promote the Con A-, LPS-, and OVA-stimulated splenocyte proliferation in the immunized mice. The results indicated that AEPS 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.

Table 2
The mRNA expression level of cytokines and transcription factors in splenocytes from the OVA-immunized mice.

Gene	OVA	OVA–Quil A	OVA–AEPS		
			(25 μg)	(50 μg)	(100 μg)
IL-4	0.28 ± 0.02	0.48 ± 0.04[b]	0.29 ± 0.06	0.46 ± 0.05[b]	0.43 ± 0.08[a]
IL-10	0.34 ± 0.02	0.70 ± 0.02[c]	0.43 ± 0.02[b]	0.66 ± 0.01[c]	0.47 ± 0.03[c]
GATA-3	0.40 ± 0.03	0.66 ± 0.04[c]	0.46 ± 0.02	0.55 ± 0.01[b]	0.58 ± 0.05[b]
IL-2	0.57 ± 0.01	0.92 ± 0.01[c]	0.58 ± 0.01	1.00 ± 0.01[c]	0.81 ± 0.00[c]
IFN-γ	0.52 ± 0.01	0.91 ± 0.01[c]	0.87 ± 0.01[c]	1.05 ± 0.02[c]	0.92 ± 0.05[c]
T-bet	0.47 ± 0.04	0.94 ± 0.01[c]	0.63 ± 0.07[a]	0.87 ± 0.04[c]	0.73 ± 0.01[c]

[a] Significant differences with OVA alone group were designated as $P < 0.05$.
[b] $P < 0.01$.
[c] $P < 0.001$.

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 [54,55]. The different Th1 and Th2 immune response profiles correspond to the activation of two distinct major subsets of T-cells characterized by their pattern of cytokine production [56]. 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. 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 [57]. Immunity to different infectious agents requires distinct types of immune responses. The Th1 response, correlated with the induction of cell-mediated immunity [58], is required for protective immunity against intracellular infectious agents, such as viruses, certain bacteria and protozoa, and presumably against cancer cells [59,60]. Th2 immunity, which control the humoral immune response through the triggering of B cell proliferation and differentiation [61], is effective for protection against most bacterial as well as certain viral infections [62,63]. In this study, the adjuvant activity of AEPS on the humoral immune responses to OVA was also evaluated. While OVA alone induced low levels of IgG1, IgG2a, and IgG2b antibodies, the addition of AEPS to OVA resulted in dramatic increases in IgG1, IgG2a, and IgG2b antibody titers. Thus, in addition to enhancing the magnitude of antibody responses, AEPS also modulated the quality of the immune responses, and elicited a balanced Th1/Th2 immune responses to OVA in mice as indicated by the significant increases in both IgG1, IgG2a and IgG2b antibody isotypes [64,65].

In order to clearly establish that Th cell-derived cytokines were involved in the adjuvant activity of AEPS, we analyzed the Th1/Th2 cytokine secretion profiles in OVA-immunized mice using ELISA. AEPS not only significantly increased the production of Th2 cytokines IL-10, but also strongly enhanced the production of Th1 cytokines IL-2 and IFN-γ from splenocytes in the OVA-immunized mice. In order to further elucidate the mechanism responsible for the changes in the amounts of Th1/Th2 cytokines, we utilized RT-PCR to analyze the mRNA expression of IL-2 and IFN-γ, the archetypal Th1 cytokine, and IL-4 and IL-10, the signature Th2 cytokine in splenocytes of the immunized mice. AEPS not only enhanced the mRNA expression of IL-4 and IL-10, but increased that of IL-2 and IFN-γ. Cytokine mRNA levels were for the most part transcriptionally regulated and selective expression of transcription factors T-bet and GATA-3 was correlated with cytokine gene and protein expression [66–68], so we measured T-bet and GATA-3 mRNA expression in splenocytes from the immunized mice. AEPS also enhanced the mRNA expression of T-bet and GATA-3 in splenocytes. These results suggested the effects of AEPS on Th1 and Th2 immune responses may result, at least in part, from the regulation of mRNA expression of the cytokines and transcription factors.

NK cells and CTL represent two major populations of cytotoxic lymphocytes [69,70], and are important in the defense against tumors and viruses [71–73]. With spontaneous cell-mediated cytotoxicities, NK cells are also functionally similar to CTLs. NK cells are able to deliver a response immediately after recognizing specific signals, including stress signals, 'danger' signals or signals from molecules of foreign origin [74]. NK cells can react against and destroy target cell without prior sensitization to it. The target cell can be a cancer cell cultured in vitro or from another tissue. NK cell activity assay is a routine method for analysis of a patient's cellular immune response in vitro, and can also be used to test the antitumor activities of possible drugs [75]. In this investigation, AEPS significantly enhanced the lytic activity of NK cells in OVA-immunized mice, suggesting that AEPS could improve cytolytic activities against autologous tumor cells and viruses.

In conclusion, these results suggested that AEPS had immunological adjuvant activity on a specific cellular and humoral immune responses to OVA in mice and could simultaneously elicits a Th1 and Th2 immune responses by regulating gene expression of Th1/Th2 cytokines and transcription factors. Taken together with its natural origin, without lethal toxicity to humans and animals, and long-standing use as folk medicines, AEPS may be a safe and efficacious adjuvant candidate suitable for a wide spectrum of prophylactic and therapeutic vaccines for which a balanced and potent stimulation of both the cellular and humoral responses is required. The studies on AEPS 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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References

[1] Lima KM, dos Santos SA, Rodrigues Jr JM, Silva CL. Vaccine adjuvant: it makes the difference. Vaccine 2004;22(19):2374–9.
[2] O'Hagan DT, Mackichan ML, Singh M. Recent developments in adjuvants for vaccines against infectious diseases. Biomol Eng 2001;18(3):69–85.
[3] Rock KL, Hearn A, Chen CJ, Shi Y. Natural endogenous adjuvants. Springer Semin Immunopathol 2005;26(3):231–46.
[4] Storni T, Kündig TM, Senti G, Johansen P. Immunity in response to particulate antigen-delivery systems. Adv Drug Deliv Rev 2005;57(3):333–55.
[5] Freund J, Casals J, Hosmer E. Sensitization and antibody formation after injection of tubercle bacili and paraffin oil. Proc Soc Exp Biol Med 1937;37:509–13.
[6] Kensil CR, Kammer R. QS-21: a water-soluble triterpene glycoside adjuvant. Exp Opin Invest Drugs 1998;7(9):1475–82.
[7] Skene CD, Sutton P. Saponin-adjuvanted particulate vaccines for clinical use. Methods 2006;40(1):53–9.
[8] Gupta RK, Relyveld EH, Lindblad EB, Bizzini B, Ben-Efraim S, Gupta CK. Adjuvants: a balance between toxicity and adjuvanticity. Vaccine 1993;11(3):293–306.
[9] Kensil CR, Patel U, Lennick M, Marciani D. Separation and characterization of saponins with adjuvant activity from *Quillaja saponaria* Molina cortex. J Immunol 1991;146(2):431–7.
[10] Kensil CR, Wu JY, Soltysik S. Structural and immunological characterization of the vaccine adjuvant QS-21. Pharm Biotechnol 1995;6:525–41.
[11] Waite DC, Jacobson EW, Ennis FA, Edelman R, White B, Kammer R, et al. Three double-blind, randomized trials evaluating the safety and tolerance of different formulations of the saponin adjuvant QS-21. Vaccine 2001;19(28/29):3957–67.
[12] Janeway Jr CA. The immune system evolved to discriminate infectious nonself from noninfectious self. Immunol Today 1992;13(1):11–6.
[13] Byars N, Allison A. Immunologic adjuvants: general properties, advantages, and limitations. In: Zola H, editor. Laboratory methods in immunology. CRC Press; 1990. p. 39–51.
[14] Allison AC, Byars NE. Immunological adjuvants and their mode of action. Biotechnology 1992;20:431–49.
[15] Pascual DM, Morales RD, Gil ED, Muñoz LM, López JE, Casanueva OLJ. Adjuvants: present regulatory challenges. Vaccine 2006;24(S2):S88–9.
[16] HogenEsch H. Mechanisms of stimulation of the immune response by aluminum adjuvants. Vaccine 2002;20(S3):S34–9.
[17] Schirmbeck R, Melber K, Kuhrber A, Janowicz ZA, Reimann J. Immunization with soluble hepatitis B virus surface protein elicits murine H-2 class I-restricted $CD8^+$ cytotoxic T lymphocyte responses in vivo. J Immunol 1994;152:1110–9.
[18] Petrovsky N. Novel human polysaccharide adjuvants with dual Th1 and Th2 potentiating activity. Vaccine 2006;24(S2):S26–9.
[19] Schepetkin IA, Quinn MT. Botanical polysaccharides: macrophage immunomodulation and therapeutic potential. Int Immunopharmacol 2006;6:317–23.
[20] Kong XF, Hu YL, Rui R, Wang DY, Li XR. Effects of Chinese herbal medicinal ingredients on peripheral lymphocyte proliferation and serum antibody titer after vaccination in chicken. Int Immunopharmacol 2004;7:975–82.
[21] Wang DY, Hu YL, Sun JL, Kong XF, Zhang BK, Liu JG. Comparative study on adjuvanticity of compound Chinese herbal medicinal ingredients. Vaccine 2005;23:3704–8.
[22] Popov SV, Golovchenko VV, Ovodova RG, Smirnov VV, Khramova DS, Popova GY, et al. Characterisation of the oral adjuvant effect of lemnan, a pectic polysaccharide of *Lemna minor* L. Vaccine 2006;24(26):5413–9.
[23] Qiu Y, Hu YL, Cui BA, Zhang HY, Kong XF, Wang DY, et al. Immunopotentiating effects of four Chinese herbal polysaccharides administered at vaccination in chickens. Poult Sci 2007;86:2530–5.
[24] Dong SF, Chen JM, Zhang W, Sun SH, Wang J, Gu JX, et al. Specific immune response to HBsAg is enhanced by β-glucan oligosaccharide containing an α-(1→3)-linked bond and biased towards M2/Th2. Int Immunopharmacol 2007;7(6):725–33.

[25] Lu Y, Wang DY, Hu YL, Huang XY, Wang JM. Sulfated modification of epimedium polysaccharide and effects of the modifiers on cellular infectivity of IBDV. Carbohydr Polym 2008;2:180–6.
[26] Huang XY, Hu YL, Zhao XN, Lu Y, Wang JM, Zhang F, et al. Sulfated modification can enhance the adjuvanticity activity of *Astragalus* polysaccharide for ND vaccine. Carbohydr Polym 2008;2:303–8.
[27] Sun YX, Liu JC. Adjuvant effect of water-soluble polysaccharide (PAP) from the mycelium of *Polyporus albicans* on the immune responses to ovalbumin in mice. Vaccine 2008;26(31):3932–6.
[28] da Silva BP, de Medeiros Silva G, Parente JP. Chemical properties and adjuvant activity of a galactoglucomannan from *Acrocomia aculeata*. Carbohydr Polym 2009;75(3):380–4.
[29] Guo ZH, Hu YL, Wang DY, Ma X, Zhao XN, Zhao BK, et al. Sulfated modification can enhance the adjuvanticity of lentinan and improve the immune effect of ND vaccine. Vaccine 2009;27(5):660–5.
[30] Nishiyama I, Fukuda T, Oota T. Genotypic differences in chlorophyll, lutein, and β-carotene. J Agric Food Chem 2005;53:6403–7.
[31] Zhao YP, Wang XY, Wang ZC, Lu Y, Fu CX, Chen SY. Essential oil of *Actinidia macrosperma*, a catnip response kiwi endemic to China. J Zhejiang Univ Sci B 2006;7(9):708–12.
[32] Jiangsu New Medical College. Dictionary of Chinese Materia Medica. Shanghai: Shanghai People's Press; 1977. p. 440.
[33] Lin SQ, Yu P, Zhu SM, Wu XC, Yu BS. Preliminary study on anticancer effect and affecting mouse immune function of crude extracts from the *Actinintia eriantha* Benth root. J Fujian Normal Univ (Nat Sci Ed) 1987;3(2):108–10.
[34] Xu HS, Wu YW, Sun HX, Yao L. Antitumor and immunomodulatory activity of polysaccharide from the roots of *Actinidia eriantha*. J Ethnopharmacol; submitted for publication.
[35] Larsen ST, Nielsen GD. The adjuvant effect of di-(2-ethylhexyl) phthalate is mediated through a PPARα-independent mechanism. Toxicol Lett 2007;170(3):223–8.
[36] Sun HX, Xie Yong, Ye YP. Advances in saponin-based adjuvants. Vaccine 2009;27(12):1787–96.
[37] Xie Y, Ye YP, Sun HX, Li D. Contribution of the glycidic moieties to the haemolytic and adjuvant activity of platycodigenin-type saponins from the root of *Platycodon grandiflorum*. Vaccine 2008;26(27):3450–8.
[38] Xie Y, Sun HX, Duo L. Platycodin D is a potent adjuvant of specific cellular and humoral immune responses against recombinant hepatitis B antigen. Vaccine 2009;27(5):757–64.
[39] Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. Colorimetric method for determination of sugars and related substances. Anal Chem 1956;28: 350–6.
[40] Bitter T, Muir HM. A modified uronic acid carbazole reaction. Anal Biochem 1962;4:330–4.
[41] Mawhinney TP, Feather MS, Barbero GJ, Martinez JR. The rapid, quantitative determination of neutral sugars (as aldononitrile acetates) and amino sugars (as O-methyloxime acetates) in glycoproteins by gas–liquid chromatography. Anal Biochem 1980;101:112–7.
[42] Sun HX, Ye YP, Pan HJ, Pan YJ. Adjuvant effect of *Panax notoginseng* saponins on the immune responses to ovalbumin in mice. Vaccine 2004;22:3882–9.
[43] Tu J, Sun HX, Ye YP. Immunomodulatory and antitumor activity of triterpenoid fractions from the rhizomes of *Astilbe chinensis*. J Ethnopharmacol 2008;119(2):266–71.
[44] Barr TA, Carlring J, Heath AW. Co-stimulatory agonists as immunological adjuvants. Vaccine 2006;24:3399–407.
[45] Marciani DJ. Vaccine adjuvants: role and mechanisms of action in vaccine immunogenicity. Drug Discov Today 2003;8:934–43.
[46] Edelman R. Vaccine adjuvants. Rev Infect Dis 1980;2(3):370–83.
[47] Aguilar JC, Rodríguez EG. Vaccine adjuvants revisited. Vaccine 2007;25: 3752–62.
[48] Sun HX. Adjuvant effect of *Achyranthes bidentata* saponins on specific antibody and cellular response to ovalbumin in mice. Vaccine 2006;24(17):3432–9.
[49] Yang ZG, Sun HX, Fang WH. Haemolytic activities and adjuvant effect of *Astragalus membranaceus* saponins on the immune responses to ovalbumin in mice. Vaccine 2005;23(44):5196–203.
[50] Sun HX. Haemolytic activities and adjuvant effect of *Bupleurum chinense* saponins on the immune responses to ovalbumin in mice. Vaccine 2006;24(9):1324–31.
[51] Sun HX, Pan HJ. Immunological adjuvant effect of *Glycyrrhiza uralensis* saponins on the immune responses to ovalbumin in mice. Vaccine 2006;24(11):1914–20.
[52] Xie Y, Pan HJ, Sun HX, Li D. A promising balanced Th1 and Th2 directing immunological adjuvant, saponins from the root of *Platycodon grandiflorum*. Vaccine 2008;26(31):3937–45.
[53] Sun HX, Zheng QF. Haemolytic activities and adjuvant effect of *Gynostemma pentaphyllum* saponins on the immune responses to ovalbumin in mice. Phytother Res 2005;19(10):895–900.
[54] Gupta RK, Siber GR. Adjuvants for humans vaccines: current status, problems and future prospects. Vaccine 1995;13:1263–6.
[55] Audibert FM, Lise LD. Adjuvants: current status, clinical perspectives and future prospects. Immunol Today 1993;14:281–4.
[56] Sedlik C. Th1 and Th2 subsets of T lymphocytes: characteristics, physiological role and regulation. Bull Inst Pasteur 1996;94:173–200.
[57] Livingston PO, Adluri S, Helling F, Yao TJ, Kensil CR, Newman MJ, et al. Phase I trial of immunological adjuvant QS-21 with a GM2 ganglioside-keyhole limpet haemocyanin conjugate vaccine in patients with malignant melanoma. Vaccine 1994;12(14):1275–80.
[58] Cher DJ, Mosmann TR. Two types of murine helper T-cell clones. II. Delayed-type hypersensitivity is mediated by Th1 clones. J Immunol 1987;138:3688–94.
[59] Mosmann TR, Sad S. The expanding universe of T-cells subsets: Th1, Th2 and more. Immunol Today 1996;17:138–46.
[60] Mahon BP, Katrak K, Nomoto A, Macadam AJ, Minor PD, Milles KHG. Poliovirus-specific $CD4^+$ Th1 clones with both cytotoxic and helper activity mediate protective humoral immunity against a lethal poliovirus infection in transgenic mice expressing the human poliovirus receptor. J Exp Med 1995;181:1285–92.
[61] Coffman RL, Seymour BWP, Lebman DA, Hiraki DD, Christiansen JA, Shrader B, et al. The role of helper T-cell products in mouse B-cell differentiation and isotype regulation. Immunol Rev 1988;102:5–28.
[62] Constant SL, Bottomly K. Induction of Th1 and Th2 $CD4^+$ T cell responses: the alternative approaches. Annu Rev Immunol 1997;15:297–322.
[63] McKee AS, Munks MW, Marrack P. How do adjuvants work? Important considerations for new generation adjuvants. Immunity 2007;27(5):687–90.
[64] Finkelman FD, Holmes J, Katona IM, Urban Jr JF, Beckmann MP, Park LS, et al. Lymphokine control of in vivo immunoglobulin isotype selection. Annu Rev Immunol 1990;8:303–33.
[65] Germann T, Bongartz M, Dlugonska H, Hess H, Schmitt E, Kolbe L, et al. Interleukin-12 profoundly up-regulates the synthesis of antigen-specific complement-fixing IgG2a, IgG2b and IgG3 antibody subclasses in vivo. Eur J Immunol 1995;25(3):823–9.
[66] Zheng W, Flavell RA. The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. Cell 1997;89(4):587–96.
[67] Szabo SJ, Kim ST, Costa GL, Zhang X, Fathman CG, Glimcher LH. A novel transcription factor, T-bet, directs Th1 lineage commitment. Cell 2000;100(6):655–69.
[68] Chakir H, Wang H, Lefebvre DE, Webb J, Scott FW. T-bet/GATA-3 ratio as a measure of the Th1/Th2 cytokine profile in mixed cell populations: predominant role of GATA-3. J Immunol Methods 2003;278(1/2):157–69.
[69] Kos FJ, Engleman EG. Immune regulation: a critical link between NK cells and CTLs. Immunol Today 1996;17:174–6.
[70] Medzhitov R, Janeway Jr CA. Innate immunity: impact on the adaptive immune response. Curr Opin Immunol 1997;9:4–9.
[71] Boon T, Cerottini JC, Van den Eynde B, van der Bruggen P, Van Pel A. Tumor antigens recognized by T lymphocytes. Annu Rev Immunol 1994;12:337–65.
[72] Moretta L, Bottino C, Cantoni C, Mingari MC, Moretta A. Human natural killer cell function and receptors. Curr Opin Pharmacol 2001;1:387–91.
[73] Rappuoli R. Bridging the knowledge gaps in vaccine design. Nat Biotechnol 2007;25:1361–6.
[74] Orange JS. Formation and function of the lytic NK-cell immunological synapse. Nat Rev Immunol 2008;8:713–25.
[75] Zhang J, Sun R, Wei HM, Tian ZG. Antitumor effects of recombinant human prolactin in human adenocarcinoma-bearing SCID mice with human NK cell xenograft. Int Immunopharmacol 2005;5:417–25.