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Immunological adjuvant effect of *Japanese ginseng* saponins (JGS) on specific antibody and cellular response to ovalbumin and its haemolytic activities

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ABSTRACT

In this study, the saponins (JGS) extracted from the rhizoma of *Japanese ginseng* were evaluated for their haemolytic activities and their potential ability as adjuvants on the immune responses to ovalbumin (OVA) in mice. The haemolytic activity of JGS was determined using 0.5% rabbit red blood cell, with its HD_{50} value being 177.78 ± 6.77 μg/mL. ICR mice were immunized subcutaneously with OVA 100 μg alone or with OVA 100 μg dissolved in saline containing Alum (200 μg), QuilA (10 and 20 μg) or JGS (50, 100 or 200 μg) on Days 1 and 15. Two weeks later (Day 28), concanavalin A (ConA)-, lipopolysaccharide (LPS)-, OVA-stimulated splenocyte proliferation and OVA-specific antibodies in serum were measured. JGS significantly enhanced the ConA-, LPS-, and OVA-induced splenocyte proliferation in the OVA-immunized mice especially at a dose of 100 μg ($P < 0.05$ or $P < 0.01$). The OVA-specific IgG, IgG1 and IgG2b antibody levels in serum were also significantly enhanced by JGS compared with OVA control group ($P < 0.01$). The results suggest that JGS showed a slight haemolytic effect and enhanced significantly a specific antibody and cellular response against OVA in mice.

1. Introduction

With the progress of new generations of vaccines, adjuvants are very important for purified, subunit and synthetic vaccines, which are likely to be less reactogenic and immunogenic than traditional vaccines [1]. Therefore, there is an urgent need for potent adjuvants to appear. Unfortunately, potent adjuvant action is often correlated with increased toxicity [2,3]. Thus, minimizing toxicity remains as one of the major challenges in adjuvant research. Aluminium salts have been the most widely used adjuvants in humans [4]. Unfortunately, Alum salts are relatively poor adjuvants in many situations, particularly at inducing cellular immune response [5–7]. QuilA are able of stimulating both the Th1 immune response and the production of cytotoxic T-lymphocyte against exogenous antigens, which makes them ideal for use in vaccines directed against both pathogens and cancer [8,9]. However, their high toxicity, haemolytic effect and instability have restricted their use in human vaccination [10–14].

Japanese ginseng grows wild throughout Japan and the southwest region of China, which has been used as a substitute for Ginseng roots. In Japan, its rhizome is used to promote the functional activity of the stomach and as an expectorant and antitussive agent [15], while in China it is used as a tonic, antiinflammatory, and haemostatic agent [16]. Its main constituents include saponins, polysaccharides, volatile oil and amine acid [17]. The pharmacological studies on this plant proved the triterpene saponins to be the main bioactive principles. The saponins from *J. ginseng* include about 15 saponins, which are divided into oleanolic acid-type and dammarane-type saponins. They are all composed of a molecule of triterpene aglycone and 1–4 molecules of glucuronic acid, glucose, arabinose or xylose [17–21] (Fig. 1). However, so far there is no available literature published about the adjuvant activity of *J. ginseng* saponins (JGS).

So in this study, the haemolytic activity of JGS and the evaluation of the adjuvant activity of JGS on the humoral and cellular immune responses to mice subcutaneously immunized with ovalbumin were first reported.

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2. Materials and methods

2.1. Materials

Ovalbumin (OVA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), concanavalin A (ConA), lipopolysaccharide (LPS), and rabbit anti-mouse IgG peroxidase conjugate were from Sigma Chemical Co. QuilA was from NOR-VET Aps. Medium RPMI-1640 was purchased from Gibco Invitrogen Co. The RMPI-1640 medium, used for immunological tests, was supplemented with HEPES buffer 10 μmol/mL, penicillin 100 IU/mL, streptomycin 100 μg/mL, L-glutamine 2 μmol/mL, 2-mercaptoethanol 50 μmol/L and 10% newborn bovine serum, pH 7.2. Goat anti-mouse IgG1 and IgG2b peroxidase conjugate were from Southern Biotech. Fetal calf serum (FCS) was provided by Hangzhou Sijiqing Corp. Aluminum hydroxide gel (Alum) was from Zhejiang Wanma Pharm. Co. All other reagents were of grade AR.

2.2. Experimental animals

Male ICR mice (GradeII, 5–6 weeks old) weighing 18–22 g, were purchased from Pharmacology Experimental Center of Jilin University and acclimatized for 1 week prior to use. All mice were housed under standard conditions at 24 ± 1 °C, with humidity of 50 ± 10%, and a 12/12 h light/dark cycle. Rodent laboratory chow pellets and tap water were supplied ad libitum. All the procedures conducted by Institute for Experimental Animals of Jilin University were carried out in strict accordance with the PR China legislation on the use and care of laboratory animals and were approved by the university committee for animal experiments.

2.3. Samples and preparation of saponins

Japanese. ginseng were purchased from a drugstore in Changchun, and identified by Professor Da-you Liu at Changchun University of Chinese Medicine according to China Pharmacopoeia (CP). The powered samples (1 kg) were extracted with 60% EtOH three times under reflux for 2 h, and then concentrated in vacuum (40 °C) to evaporate the solvent to give a small volume. After extracting with ether (3× 0.5 L), the water layer portion was extracted with water-saturated *n*-BuOH. The *n*-BuOH solution was concentrated and dried in vacuum (50 °C). The dried extract was subjected to D101–D941 coupled resin column chromatography, washed with H_2O, and then eluted with 60% EtOH. From 60% EtOH elution we get about 20.12 g of JGS. JGS contained 83.7 ± 2.17% of saponins determined by the spectrophotometric method at 538 nm estimated with reference to a calibration curve made from a set of oleanolic acid [22]. A stock JGS solution with a concentration of 2 mg/mL was prepared by dissolving in 0.9% saline and sterilized by passing it through a 0.22 μm millipore filter. In order to avoid false positive of the immunological test of the saponins, contaminant endotoxins, which possess mitogenic activity, were analyzed by a gel-clot *Limulus* amebocyte lysate assay. The endotoxin level in the JGS solution was less than 0.5 EU (endotoxin units)/mL.

2.4. Haemolytic activity assay

The haemolytic activity of *J. ginseng* or QuilA was measured according to the method of Sun et al. [23]. New Zealand rabbit was purchased from Jilin Experimental Animal Center, China. Aliquots of 7 mL of rabbit blood collected in Alsevers (Hemostat, Dixon, CA) were washed three times with sterile saline solution (0.9%, w/v, NaCl, pyrogen free) by centrifugation at 180 × *g* for 5 min. The cell suspension was prepared by finally diluting the pellet to 0.5% in saline solution. A volume of 0.5 mL of the cell suspension was mixed with 0.5 mL diluents containing 5, 10, 25, 50, 100, 250, 500 and 1000 μg/mL concentrations of JGS in saline solution. The mixtures were incubated for 30 min at 37 °C and centrifuged at 70 × *g* for 10 min. The free haemoglobin in the supernatants was measured spectrophotometrically at 412 nm. Saline and distilled water were included as minimal and maximal haemolytic controls. The haemolytic percent developed by the saline control was subtracted from all groups. Each experiment included triplicates at each concentration.

2.5. Immunization

To examine the adjuvant activities of Alum, QuilA and JGS, Ovalbumin (OVA) was used as model antigen. Male ICR mice were divided into eight groups, each consisting of five mice. Animals were immunized subcutaneously on the back using OVA 100 μg alone or OVA 100 μg dissolved in saline containing Alum (200 μg), QuilA (10 and 20 μg), or JGS (50, 100 and 200 μg) on Day 1. Saline-treated animals were included as controls. Immunizations were performed twice at a 14-day interval and mice were sacrificed 14 days after the second immunization. Sera and splenocytes samples collected from animals on Day 28 were used for proliferation assay, measurement of anti-OVA IgG, IgG1 and IgG2b by ELISA [24].

2.6. Splenocyte proliferation assay in vivo

The OVA-immunized mice were sacrificed by cervical dislocation and spleens were collected under aseptic conditions in RPMI-1640. Spleen cells of mice were prepared by gently mincing and grinding the spleen fragment in RPMI-1640 medium on a fine steel mesh. To isolate mononuclear cells, 5 mL aliquots of the spleen cell suspension were layered onto 2.5 mL aliquots of a polysucrose–sodium ditrizoate solution and centrifuged at 300 × *g* at 4 °C for 10 min. Mononuclear cells were gently removed from the interface between medium and histopaque and transferred to a sterile container and washed with RPMI-1640. At last, the cells were resuspended in 5 mL RPMI-1640 medium, and cell numbers were done with a haemocytometer by trypan blue dye exclusion technique. Cell viability exceeded 95%. Splenocyte proliferation was assayed as previously described [25]. Briefly, an aliquot of 100 μL of splenocytes at 5×10^6 cells/mL was seeded into each well of a 96-well flatbottom microtiter plate, thereafter ConA (final concentration 5.0 μg/mL), LPS (final concentration 10.0 μg/mL), OVA (final concentration 10.0 μg/mL), or medium were added giving a final volume of 200 μL. After preincubation for 68 h at 37 °C in a humidified 5% CO_2 incubator, 50.0 μL of 0.4% MTT was added into each well. The plate was incubated for another 4 h and centrifuged (1400 × *g*, 5 min) to remove the untransformed MTT carefully by pipetting. Then to each well a total of 200 μL DMSO was added to fully dissolve the colored material. The absorbance at 570 nm with a 630 nm reference was measured on an ELISA reader (Model 680, Bio-RAD Instruments). 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. Each experiment was performed in triplicate.

2.7. Measurement of OVA-specific antibody

OVA-specific IgG, IgG1 and IgG2b antibodies in serum were detected by ELISA according to the method previously described by Sjoelander et al. with some modifications [26]. In brief, the wells of 96-well microtiter plates were coated with 100 μL of OVA solution