



Enhancement of immune responses to influenza vaccine (H3N2) by ginsenoside Re

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ABSTRACT

This study was designed to evaluate the adjuvant effect of ginsenoside Re isolated from the root of *Panax ginseng* on the immune responses elicited by split inactivated H3N2 influenza virus antigen in a mouse model. Forty-eight ICR mice were randomly distributed into six groups with 8 mice in each group. All animals were subcutaneously (s.c.) immunized twice on weeks 0 and 3 with 50 µg Re, inactivated H3N2 influenza virus antigen equivalent to 10 or 100 ng of hemagglutinin (HA) or inactivated H3N2 influenza virus antigen equivalent to 10 ng HA adjuvanted with Re (25, 50 or 100 µg). Two weeks after the boost, blood samples were collected for measurement of serum IgG, the IgG isotypes and HI titers. Splenocytes were separated for the detection of lymphocyte proliferation and production of IFN-γ and IL-5 *in vitro*. Results showed that co-administration of Re significantly enhanced serum specific IgG, IgG1, IgG2a and IgG2b responses, HI titers, lymphocyte proliferation responses as well as IFN-γ and IL-5 secretions, indicating that both Th1 and Th2 were activated. Considering the adjuvant effect demonstrated in this study, Re deserve further studies for improving the quality of vaccines where mixed Th1/Th2 immune responses are needed.

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1. Introduction

Influenza viruses belong to the *Orthomyxoviridae* family and are the major cause of respiratory disease in humans. Previously, outbreaks in humans due to influenza types A and B were responsible for substantial mortality and morbidity: 200,000 hospitalizations and 36,000 deaths annually in the U.S. (www.cdc.gov), particularly in high risk groups, such as the elderly, infants, and those with chronic underlying medical conditions [1]. Influenza infections in the elderly often lead to secondary bacterial infection that results in severe symptoms and occasionally death [2–4]. Furthermore, the highly pathogenic avian influenza virus H5N1 strain has caused outbreaks of disease in domestic poultry in Asian countries, and the unprecedented spread of the H5N1 influenza virus has been associated with several human infections and deaths in Vietnam, Thailand, Indonesia, and China [1,5–10].

Influenza vaccines have effectively been used for prevention of influenza infections. Nichol et al. [11,12] have reported that influenza vaccines have decreased the number of cases requiring hospitalization due to any type of respiratory condition. In a study using experimental animals, influenza vaccine has effectively protected host animals from severe influenza–bacteria superinfectious diseases [13]. In spite of the high efficacy of inactivated vaccines against influenza, the efficacy of inactivated influenza vaccines used widely is reduced in elderly people

and infants [14–16], and these vaccines protect poorly against drift variants of influenza virus [17,18]. At the same time, the use of some adjuvants with inactivated influenza vaccines not only strengthens their immunogenicity and protective efficiency, but also increases their ability to protect from drift variants of influenza virus. The potential threat of an influenza pandemic has led to studies on the development of new effective and safe adjuvants. An optimal adjuvant should not only increase immunogenicity and effectiveness of a vaccine, but also should be cheap to produce, non-toxic, biodegradable, biocompatible, immunologically inert, stable, and with long shelf life. It should promote not only humoral immunity, but also induce cellular immune response without inducing IgE antibodies (i.e., be non-allergenic) [19,20].

Panax ginseng C. A. Meyer as a traditional medicine has been utilized in China for at least 2000 years [21]. The drug has been believed to stimulate the natural resistance against infections [22]. Ginseng saponins (GS), or ginsenosides, are believed to be the pharmacologically active substances in total ginseng extracts. Recent studies on ginseng saponins have demonstrated that GS has adjuvant activity capable of boosting both cellular (Th1) as well as humoral (Th2) immune responses [23–27]. Ginsenosides are mainly chemically triterpenoid glycosides of the dammarane series. At present, more than 30 ginsenosides have been identified in *P. ginseng* [28]. The adjuvant activities of ginsenosides with different molecular structures are dependent mainly on the sugar side chains attached to their dammarane skeleton. More recent investigation has found that the adjuvant activity of ginsenosides Rg1, Re, Rg2, Rg3 and Rb1 from the root of *Panax ginseng* is more potent than that of Rd, Rc and Rb2 [29]. Chemical analysis has shown that Re is found not only in the root but

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also in the stem and leaf of *P. ginseng* [30]. This discovery has greatly decreased the cost of Re production. Present study was designed to evaluate the adjuvant properties of ginsenoside Re by measuring serum specific antibody responses, hemagglutination inhibition titers (HI), lymphocyte proliferation as well as cytokine production by splenocytes in mice immunized with inactivated H3N2 influenza vaccines in combination with Re.

2. Materials and methods

2.1. Animals

Female ICR mice were purchased from Shanghai Laboratory Animal Center (SLAC) Co. Ltd. (Shanghai, China), and housed in polypropylene cages with sawdust bedding in hygienically controlled environment. Feed and water were supplied *ad libitum*. All procedures related to the animals and their care conformed to the internationally accepted principles as found in the Guidelines for Keeping Experimental Animals issued by the government of China.

2.2. Antigen and adjuvant

Split inactivated influenza virus A/Fujian/411/2002 (H3N2) strain was kindly supplied by Zhejiang Provincial Center for Diseases Control and Prevention, which contained 326 µg/ml of hemagglutinin (HA). Ginsenoside Re extracted from the root of *Panax ginseng* C.A. Meyer was purchased from Hongjiu Ginseng Industry Co. Ltd (Jilin, China). Re was white powder with purity of 98% and molecular weight at 947. Re was first dissolved in dimethyl sulfoxide (DMSO), then diluted with physiological saline solution (1000 µg/ml) and sterilized by passing through a 0.22 µm filter. The endotoxin level in above solutions was less than 0.5 endotoxin unit (EU)/ml by a gel-clot *Limulus* amoebocyte lysate assay (Bath no., Zhanjiang A&C Biological Ltd., Zhanjiang, China).

2.3. Immunization

Forty-eight ICR mice were randomly distributed into six groups with 8 mice each. Each of the animals was subcutaneously (s.c.) immunized twice at 3 week intervals with 200 µl of physiological saline solution containing (1) 50 µg Re; (2) 10 ng HA; (3) 100 ng HA; (4) 10 ng HA + 25 µg Re; (5) 10 ng HA + 50 µg Re or (6) 10 ng HA + 100 µg Re. Two weeks after the boost, blood samples were collected for measurement of serum HI titers using chicken red blood cells, and HA-specific IgG titers as well as IgG isotype levels. Splenocytes were prepared for determination of cellular proliferation and production of IFN-γ and IL-5.

2.4. Measurement of specific IgG and the IgG isotypes

Serum samples were analyzed for measurement of HA-specific IgG titer and IgG isotype responses by indirect enzyme-linked immunosorbent assay. All the wells of polyvinyl 96-well microtitre plates were coated with 100 µl of 1 µg/ml HA diluted in 0.05 M carbonate buffer, pH 9.6 and incubated overnight at 4 °C. After five washes with phosphate buffer saline (PBS, pH 7.2) 0.05% Tween-20 (PBST), the wells were blocked with PBS 5% skimmed milk and incubated at 37 °C for 2 h. Following five washes, 100 µl of serum, serially diluted for IgG analysis, diluted 1/1000 in PBS 5% skimmed milk for isotypes, was added to each well and incubated at 37 °C for 45 min. Plates were then washed five times in PBST. For IgG titer detection, 100 µl of goat anti-mouse IgG (1/500) (Kirkegaard, Perry Lab., Maryland, USA), was added to all wells and incubated at 37 °C for 45 min. Plates were washed again with PBST. A hundred microliters of 3,3',5,5'-tetramethylbenzidine solution (100 µg/ml of 0.1 M citrate-phosphate, pH 5.0) solution (100 µg/ml of 0.1 M citrate-phosphate, pH 5.0) was added to each well and incubated for 15 min at room temperature. The reaction was stopped

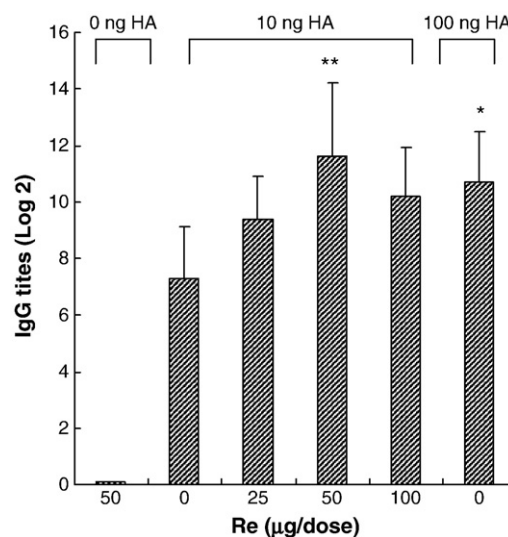


Fig. 1. Serum IgG titers elicited by inactivated H3N2 influenza virus antigen. Mice ($n = 8$) received twice subcutaneous injection at weeks 0 and 3 of 50 µg Re, inactivated H3N2 influenza virus antigen (equivalent to 10 or 100 ng HA) or inactivated H3N2 influenza virus antigen (equivalent to 10 ng HA) adjuvanted with Re (25, 50 or 100 µg). The mice were bled 2 weeks after the second immunization for analysis of IgG titers by indirect ELISA. Values above the cut-off background level, mean value of sera from saline-immunized mice (negative controls) multiplied by a factor of 2.1, were considered positive. Values represent mean \pm S.D. Titers were depicted as reciprocal end-dilutions. Significant differences with 10 ng HA groups were designated as * $P < 0.05$ and ** $P < 0.01$.

by adding 50 µl of 2 M H_2SO_4 to each well. The optical density of the plate was read by an automatic ELISA plate reader at 450 nm. Values above the cut-off background level (mean value of sera from saline-immunized mice multiplied by a factor of 2.1) were considered positive. Titers were depicted as reciprocal end-dilutions. For subclasses, 100 µl of biotin conjugated goat anti-mouse IgG1 or IgG2a or IgG2b or IgG3 (1:600 dilution, Santa Cruz Biotechnology Inc., California, USA) was added to corresponding plate and then incubated for 45 min at 37 °C. After washing, 100 µl of horseradish peroxidase conjugated anti-biotin (BD Biosciences, Pharmingen, USA) diluted 1:4000 in PBST was added to each well and incubated for 30 min at 37 °C. Incubations, washing and development were as described above for detection of HA-specific total IgG. The optical density of the plate was read at 450 nm.

2.5. Hemagglutination inhibition (HI) assay

Serum HI titers were determined according to the protocol adapted from the Center for Disease Control laboratory-based influenza surveillance manual [31]. Serum samples were serially diluted 2-fold into V-bottom 96-well microtiter plates. An equal volume of virus, adjusted to 4 HA units of antigen was added to each well. The plates were covered and incubated at room temperature for 30 min followed by the addition of freshly prepared 1% chicken erythrocytes (RBCs) in PBS. The plates were mixed by agitation, covered, and allowed to set for 60 min at 25 °C. The HI titer was determined by the reciprocal of the last dilution which contained non-agglutinated RBCs. Positive and negative serum controls were included on each plate. Mean HI titers and standard deviation were calculated for each group.

2.6. Lymphocyte proliferation assay

Spleen collected from the HA-immunized ICR mice under aseptic conditions, in Hank's balanced salt solution (HBSS, Sigma), was minced and passed through a fine steel mesh to obtain a homogeneous cell suspension. After centrifugation ($380 \times g$ at 4 °C for 10 min), the

pelleted cells were washed three times in PBS and resuspended in complete medium (RPMI 1640 supplemented with 0.05 mM 2-mercaptoethanol, 100 U/ml penicillin, 100 µg/ml streptomycin and 10% heat inactivated FCS). Cell numbers were counted with a haemocytometer by trypan blue dye exclusion technique. Cell viability exceeded 95%. Splenocyte proliferation was assayed as described previously [29] with some modification. Briefly, splenocytes were seeded into a 96-well flat-bottom microtiter plate (Nunc) at 5.0×10^6 cell/ml in 100 µl complete medium, thereafter concanavalin A (Con A, final concentration 5 µg/ml), LPS (final concentration 7.5 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% CO₂ for 2 days. All the tests were carried out in triplicate. The cell proliferation was evaluated using MTT methods. Briefly, 50 µl of MTT solution (2 mg/ml) were added to each well 4 h before the end of incubation. The plates were centrifuged (1400×g, 5 min) and the untransformed MTT was removed carefully by pipetting. To each well 150 µl of a DMSO working solution (192 µl DMSO with 8 µl 1 N HCl) was added, 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 IFN-γ and IL-5 produced by splenocytes

Single cell suspensions were adjusted to a concentration of 2.5×10^6 cells/ml in complete medium. To a 96-well flat-bottom microtiter plate (Nunc), 100 µl of the cell suspension and equal volume of Con A solution (final concentration 5 µg/ml) were added. The plates were incubated at 37 °C in a 5% CO₂ atmosphere for 48 h. After that, the culture supernatants were collected for cytokine assay. The concentrations of IFN-γ and IL-5 were determined by a commercial capture ELISA kit (R & D Systems Inc., Minneapolis, USA). Concentrations of cytokines were calculated from interpolation of the cytokine standard curve.

2.8. Statistical analysis

Data are expressed as mean ± S.D. Boniferroni method was used to compare the parameters between groups [29]. *P*-values of less than 0.05 were considered statistically significant.

3. Results

3.1. Serum specific IgG and IgG isotypes

Serum specific IgG and the IgG subclasses were measured by an indirect ELISA to evaluate the adjuvant effect of ginsenoside Re on the humoral immune responses. Fig. 1 shows that 10 ng of HA induced significantly lower HA-specific IgG titers (1:160) than 100 ng of HA (1:1659) (*P* < 0.05). However, IgG titer induced by co-administration of HA (10 ng) with Re (50 µg) was 19 times higher (1:3044) than that induced by the same dose of HA (10 ng) administered alone (*P* < 0.01) and even numerically higher than the IgG titer elicited by 100 ng of HA (*P* > 0.05). While IgG titers were higher in mice immunized with 10 ng of HA plus Re at a dose range from 25 to 100 µg than in the control, highest IgG titer was found in the group adjuvanted with 50 µg of Re. As no OD values of the sera from Re-injected mice were recorded above $2.1 \times$ mean value of the sera from saline-injected mice (negative controls), IgG titer was actually undetectable in Re-injected group.

Fig. 2 indicates that 100 ng of HA induced higher HA-specific IgG1 (*P* < 0.05), IgG2a, IgG2b (*P* < 0.05) and IgG3b responses than 10 ng of HA. Supplement of Re (25, 50, 100 µg) in 10 ng of HA enhanced the isotypes IgG1 (*P* < 0.01), IgG2a (*P* < 0.05), IgG2b (*P* < 0.05) and IgG3b

(*P* < 0.01) responses with significantly higher isotypes found in mice immunized with 10 ng HA plus 50 µg Re.

3.2. HI titers

To investigate the effect of Re on serum HI titers, mice were immunized twice s.c. and serum HI responses were determined. Fig. 3 indicates that 100 ng of HA induced numerically higher HA-specific HI titer (1:624) than 10 ng of HA (1:156) (*P* > 0.05). However, co-administration of HA (10 ng) with Re (50 µg) induced 10.8 times higher HI titer (1:1680) than the same dose of HA was administered alone (*P* < 0.01).

3.3. Lymphocyte proliferation

The effect of ginsenoside Re on splenocyte proliferative responses to Con A and LPS stimulation, and the results are shown in Fig. 4. Higher splenocyte responses were found in mice immunized with 100 ng HA to Con A (*P* < 0.05) and LPS when compared to mice immunized with 10 ng HA only. Supplement of Re (50 µg) in 10 ng HA significantly enhanced splenocyte proliferative responses to both Con A and LPS when compared to mice immunized with those with 10 ng HA alone (*P* < 0.01).

3.4. Production of IFN-γ and IL-5

After in vitro stimulation of splenocytes with Con A for 48 h, splenocytes from the mice immunized with 100 ng HA secreted significantly higher IL-5 (*P* < 0.05) but numerically higher IFN-γ (*P* > 0.05) than the control (10 ng HA) (Fig. 5). However, both IFN-γ (*P* < 0.01) and IL-5 (*P* < 0.05) were significantly higher in the cultures of the splenocytes from the mice immunized with 10 ng HA plus Re (50 µg) than those with 10 ng HA alone.

4. Discussion

Adjuvant properties of ginsenoside Re have been demonstrated for inactivated H3N2 influenza vaccines in a mouse model. Co-administration of ginsenoside Re with inactivated influenza virus A/Fujian/411/2002 (H3N2) induced significantly higher serum specific IgG and the isotype responses, HI titers, splenocyte proliferation in response to Con A and LPS as well as production of IL-5 and IFN-γ by splenocytes than in mice administered the antigen alone.

The mouse model has been used to study the immunity of a host against influenza infection. For examples, Prabakaran et al. [32] have reported that intranasal vaccination of recombinant baculovirus surface-displayed hemagglutinin (BacHA) or inactivated whole H5N1 viral vaccine with a recombinant cholera toxin B subunit (rCTB) as a mucosal adjuvant can provide 100% protection against 10MLD₅₀ of homologous and heterologous H5N1 strains. Cox et al. [33] have observed that the mice immunized with a split virus vaccine can effectively limit viral replication and this correlates high influenza specific serum IgG concentrations. The IgG response elicited by antigen is dose-dependent. In this study, the mice immunized with 100 ng HA antigen had significantly higher IgG and the IgG isotypes than the mice immunized with 10 ng HA antigen. However, supplement of ginsenoside Re (50 µg) in 10 ng HA antigen significantly amplified IgG and the IgG isotype responses as indicated in Figs. 1 and 2. Ginsenoside Re is one of the ginseng saponins identified in *P. ginseng*. Enhanced immune responses by ginseng saponins have also been found previously in other studies. For examples, Rivera et al. [23] have found an increased specific HI titers in guinea pigs immunized with co-administration of porcine parvovirus antigen with ginseng saponin; Hu et al. [24] have reported an enhanced specific IgG responses in both milk and peripheral blood of cattle vaccinated with *Staphylococcus aureus* bacterin; Song et al. [27] have

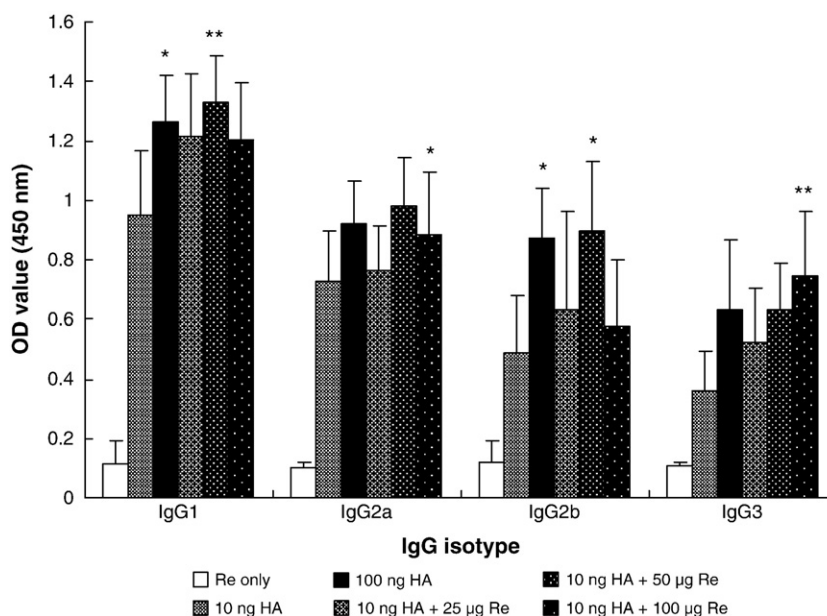


Fig. 2. Serum anti-HA IgG1, IgG2a, IgG2b and IgG3 levels after mice ($n=8$ /group) received twice subcutaneous injection at weeks 0 and 3 of 50 µg Re, inactivated H3N2 influenza virus antigen (equivalent to 10 and 100 ng HA) or inactivated H3N2 influenza virus antigen (equivalent to 10 ng HA) adjuvanted with Re (25, 50 and 100 µg). The mice were bled 2 weeks after the second immunization for IgG isotype analysis by indirect ELISA. Values represent mean \pm S.D. Significant differences with 10 ng HA groups were designated as * $P<0.05$ and ** $P<0.01$.

recently observed an enhanced IgG response in mice injected with inactivated foot-and-mouth disease virus antigen in combination with saponins isolated from ginseng stem and leaf.

Immunity to different infectious agents requires distinct types of immune responses. Defense against intracellular pathogens tends to involve Th1 type immune responses dominated by the production of IFN- γ , IgG2a antibodies, delayed type hypersensitivity (DTH) and cytotoxic T lymphocytes (CTL), while resistance to extracellular pathogens is often associated with humoral responses dominated by high levels of IgG1 and production of IL-4 and IL-5 [34]. One of the major challenges in vaccinology is the development of vaccine formulations that will induce immune responses appropriate for the particular pathogen since the wrong response could lead to increased

pathology and possibly enhanced spread of the pathogens. Thus, adjuvants can be a valuable tool for tailoring the desired immune responses. Polarized Th1 type immunity can be achieved by addition of complete Freund's adjuvant (CFA) and CpG DNA to an antigen [35,36]. In contrast, Th2 antibody responses can be enhanced by alum or incomplete Freund's adjuvant (IFA), as indicated by more IgG1 relative to IgG2a [36,37]. Some adjuvants or their combinations can promote mixed Th1/Th2 responses. For instance, purified Quillaja saponin (QS 21), a combination of CFA + IFA induce IFN- γ (Th1 type)

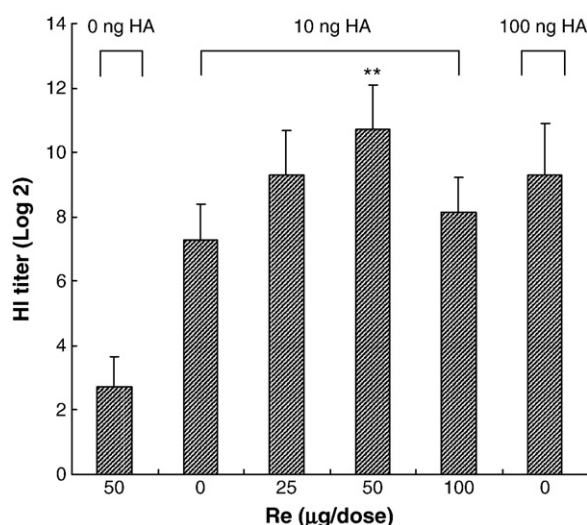


Fig. 3. Serum hemagglutination inhibition titers after mice ($n=8$ /group) received twice subcutaneous injection at weeks 0 and 3 of 50 µg Re, inactivated H3N2 influenza virus antigen (equivalent to 10 and 100 ng HA) or inactivated H3N2 influenza virus antigen (equivalent to 10 ng HA) adjuvanted with Re (25, 50 and 100 µg). The mice were bled 2 weeks after the second immunization for analysis of hemagglutination inhibition test. Values represent mean \pm S.D. Significant differences with 10 ng HA groups were designated as * $P<0.05$ and ** $P<0.01$.

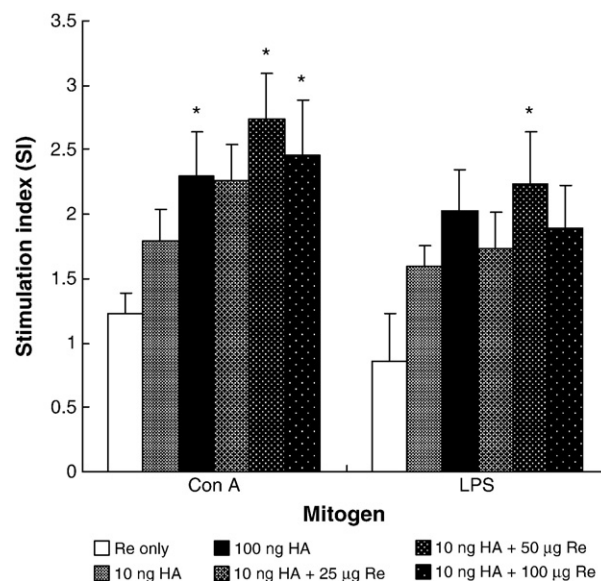


Fig. 4. Mitogen-stimulated proliferation of splenocytes isolated from mice ($n=8$) receiving twice subcutaneous injection at weeks 0 and 3 of 50 µg Re, inactivated H3N2 influenza virus antigen (equivalent to 10 and 100 ng HA) or inactivated H3N2 influenza virus antigen (equivalent to 10 ng HA) adjuvanted with Re (25, 50 and 100 µg). Splenocytes were prepared 2 weeks after the last immunization and cultured with Con A, LPS or RPMI 1640. Splenocyte proliferation was measured by the MTT method as described in the text, and shown as a stimulation index. The values were represented mean \pm S.D. Significant differences with 10 ng HA groups were designated as * $P<0.05$ and ** $P<0.01$.

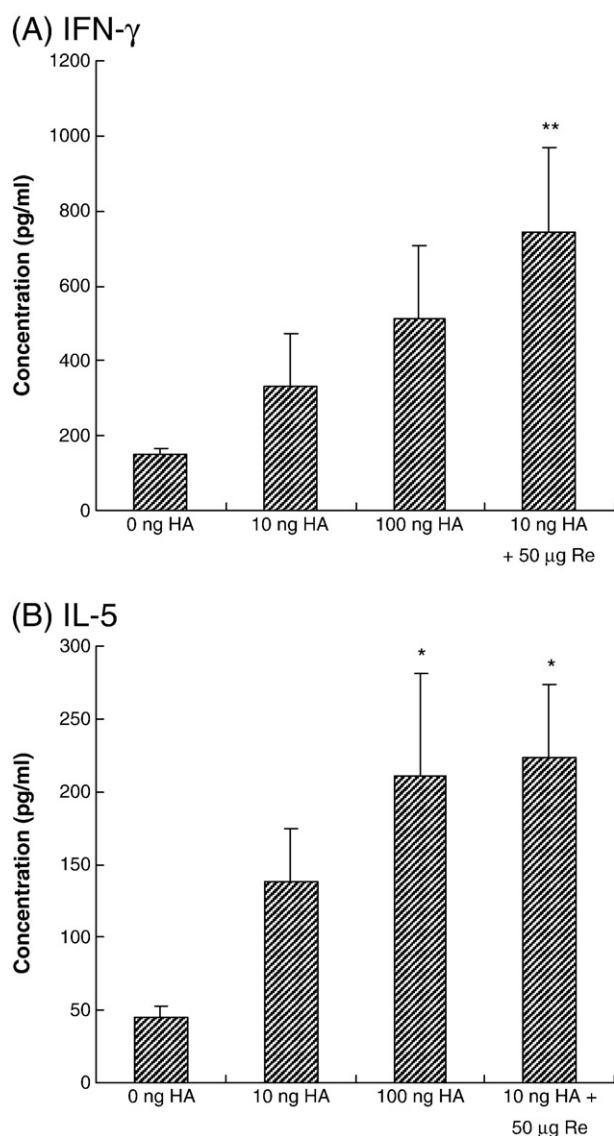


Fig. 5. IFN- γ (A) and IL-5 (B) production by mouse splenocytes stimulated with Con A mice ($n=8$) received twice subcutaneous injection at weeks 0 and 3 of 50 μ g Re, inactivated H3N2 influenza virus antigen (equivalent to 10 ng HA) adjuvanted with or without Re (50 μ g). Splenocytes were prepared 2 weeks after the last immunization and cultured with Con A (5 μ g/ml) for 48 h. The supernatants were harvested for determination of IFN- γ and IL-5 by a capture ELISA. The values are presented as mean \pm S.D. Significant differences with 10 ng HA group were designated as * $P<0.05$ and ** $P<0.01$.

and IL-4 (Th2 type) responses [37], while a liposomal formulation of leishmania antigens or ginseng stem and leaf saponin (GSLs)/oil emulsion results in mixed Th1/Th2 (both serum IgG1 and IgG2a) responses [38]. There are two types of influenza vaccines currently in use: inactivated whole virus vaccines and disrupted viral antigens containing viral surface glycoproteins [1]. As the whole virus vaccine causes significantly higher adverse reactions than does the hemagglutinin split vaccine [39], only the HA split vaccine is used in China. The split vaccine exhibits 70–90% efficacy in reducing the incidence of clinical illness but fails to prevent influenza virus infection. Subcutaneous vaccination with the HA vaccine can induce the production of virus-neutralizing antibody in the serum but not a cell-mediated immune response, including CTL activity [40,41]. According to recent observation by Bungener et al. [42], Th1 immune response plays an important role in the immunity against influenza virus infection. Our present study has demonstrated that addition of Re in influenza virus HA antigen not only can enhance IgG1 but also IgG2a responses. Such

enhanced IgG1/IgG2 responses may be attributed the increased production of IL-5 and IFN- γ as indicated in Fig. 5, suggesting that both Th1 and Th2 immune responses were stimulated. Thus, when the purpose of a vaccination is to activate both Th1 and Th2 immune response, the supplement of Re in vaccine is indicated.

Results of lymphocyte proliferation assay depend on the mitogen used. Both Con A- and LPS-induced proliferative responses were enhanced in the mice receiving co-administration of influenza virus antigen and Re. Enhanced lymphocyte response to LPS indicates that B lymphocytes were activated, indicating that both T and B lymphocytes were stimulated [43].

In conclusion, co-administration of ginsenoside Re with inactivated H3N2 influenza virus antigen in mice significantly amplified serum specific IgG and the IgG isotype responses, HI titers, lymphocyte proliferation as well as IL-5 and IFN- γ secretions, suggesting that both Th1 and Th2 immune responses were activated. Considering the adjuvant effect of Re demonstrated in this study, and a GS preparation containing Re has been licensed for injection in humans, Re deserve further studies for improving the quality of vaccines where mixed Th1/Th2 immune responses are needed.

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