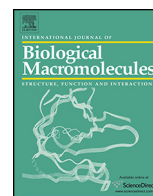




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Immunoadjuvant activity in mice of polysaccharides isolated from the leaves of *Panax ginseng* C.A. Meyer

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ABSTRACT

Our previous study showed polysaccharide (GS-P) isolated from the leaves of *Panax ginseng* C.A. Meyer possessed anti-tumor metastatic activity in mouse model. In this study, we evaluated the immunoadjuvant effect of GS-P on the induction of humoral and cellular immune responses against ovalbumin (OVA) in mice. When mice were immunized subcutaneously with OVA admixed with or without GS-P, the OVA + GS-P group showed significantly higher antibody production than the group immunized with OVA alone. This suggests that GS-P has the ability to enhance the adaptive immune response. In addition, the OVA + GS-P + FIA (Freund's incomplete adjuvant) group induced higher levels of antigen-specific IgG1 and IgG2b antibodies than the OVA + FIA group. The culture supernatant obtained from the splenocytes of mice immunized with OVA + GS-P + FIA showed higher levels of OVA-specific Th1-type (IL-2, IFN- γ , GM-CSF) and Th2-type (IL-10) cytokines. Following *in vitro* analysis of T cell proliferation, the splenocytes of mice treated with OVA + GS-P + FIA showed significantly more proliferation than those treated with OVA + FIA. Further, the production of IgE antibody was dramatically reduced when OVA + GS-P + FIA was used to immunize mice rather than OVA + FIA or OVA + FCA (Freund's complete adjuvant). Collectively, these results suggest that GS-P may possess adjuvant activity that potentially enhances humoral as well as cellular immune responses.

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1. Introduction

Immunoadjuvants are known to modulate immune cells and enhance humoral and cellular immune responses in the host. To date, aluminum-containing compounds (alum), such as hydroxide and phosphates, are the only adjuvants approved for clinical use [1]. However, alum adjuvants show some drawback such as induction of allergies according to IgE production [2]. Therefore, the development of a more effective adjuvant is needed.

The root of *Panax ginseng* C.A. Meyer (ginseng) has been used as a traditional medicine for over 2000 years in East Asia. Pharmacological and clinical studies carried out over the past 40 years have focused on the biological activities of ginseng, such as its

radioprotective, antitumor, and antiviral activities [3,4]. The major ingredients of ginseng known to modulate the immune system are ginsenosides and polysaccharides, which can elicit the activation of natural immunity [5–7] as well as the adaptive immune response in immunizations with protein antigens and ginseng as an adjuvant [8]. The abilities of ginseng extracts or its active ingredients to protect against tumors and viral infections through the activation of innate immune-related cells, such as NK cells and macrophages, are well-documented [5–7]. Results reported by Sun et al. [8] and Hu et al. [9] showed that ginseng crude extracts or its ingredients possessed adjuvant activity, enhancing Th1- and Th2-type immune responses against proteins or bacteria in mice or cattle. In addition, ginseng had a synergistic effect on the antitumor activity of the antitumor drug paclitaxel when the two were administered in mice, suggesting that ginseng can be used as an adjuvant in cancer chemotherapy [10]. In addition, River et al. [11] reported that serum antibody titers of pigs immunized with porcine parvovirus as an antigen plus alum and ginseng extract as adjuvants were higher than those of pigs immunized with the antigen plus alum alone,

Abbreviations: FIA, Freund's incomplete adjuvant; FCA, Freund's complete adjuvant; OVA, ovalbumin; s.c., subcutaneously.

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suggesting that ginseng extract has a synergistic effect with commercial adjuvants. The results of numerous studies have indicated that the biological and pharmacological effects of ginseng are concentrated in the root [5–11]. In contrast, some studies report that polysaccharides isolated from the leaves of *P. ginseng* possess biological activities, such as anti-complementary [12] and anti-ulcer [13] activities, enhancement of macrophage Fc receptor activity [14] as well as the anti-metastatic activity through enhancing of innate immune function [15]. However, there has been no report regarding the potential immunoadjuvant activity of polysaccharides isolated from the leaves of ginseng. Therefore, we investigated the ability of GS-P, the purified polysaccharides from the leaves of *P. ginseng*, to act as an adjuvant and enhance humoral and cellular immune responses specific to the ovalbumin (OVA) antigen in mice.

2. Materials and methods

2.1. Preparation of polysaccharides from ginseng leaves

Ginseng leaves were collected in Keumsan, Chungnam Province, Korea late in the fall of 2013 and dried using a conventional oven for 24 h. The isolation and purification methods of polysaccharide fraction (GS-P) from the leaves of ginseng were described previously [15]. In brief, the ginseng leaves (1000 g) were broken into pieces and then decocted with distilled water (20 L) for 6 h. The supernatant (GS-W) was collected by centrifugation at 7000 rpm for 30 min and adjusted to pH 6.0 using a diluted HCl solution. The GS-W fraction was loaded onto a Diaion HP-20 column (Mitsubishi Chemical Co. Ltd., Japan) to remove low-molecular-weight materials, and the unabsorbed fraction was collected (GS-C1). Next, GS-C1 was loaded onto a Diaion PA-312 (Mitsubishi Chemical Co. Ltd.) anion exchange column, which was subsequently washed with washing buffer (20 mM Tris-HCl buffer, pH 8.0) and eluted with 0.55 M NaCl in 20 mM Tris-HCl buffer. The eluent was concentrated by an evaporator and then precipitated by adding four volumes of EtOH (GS-C2). The GS-C2 fraction was dialyzed using a dialysis bag (molecular mass cut off: 1000; Spectrum Medical Industries Inc., USA) to remove ethanol and lyophilized to obtain the purified polysaccharide fraction GS-P.

2.2. Mice

Specific pathogen-free (SPF) mice (BALB/c, female, 5–6 weeks) were purchased from Orient Bio (Seongnam, Korea) and maintained under constant conditions (temperature 20–25 °C; humidity 40–60%; light/dark cycle 12 h) in an SPF room. Animal experiments were performed according to the guidelines of the Ethics Committee for Use of Experimental Animals at Kyonggi University (2015-003).

2.3. Immunization of mice and serum collection

Mice were immunized with OVA (Sigma, USA; 5 µg/mouse) alone, OVA+GS-P (5 µg/mouse), OVA+FCA (Freund's complete adjuvant), OVA+FIA (Freund's incomplete adjuvant), or OVA+FIA+GS-P by s.c. injection. OVA emulsified in FCA (OVA+FCA) was used as a positive control for a typical adjuvant. The mice in each group were immunized twice every 2 weeks using the same protocol. The immunized mice were bled from the retro-orbital sinus on the indicated weeks (2nd week or 5th week), and serum samples of each mouse were collected then, analyzed for antigen-specific antibody production.

2.4. Measurement of antigen-specific antibody production

Levels of antibody production in immunized mice sera were measured by enzyme-linked immunosorbent assay (ELISA). In brief, flat-bottomed 96-well plates were coated with antigen (OVA) in 50 mM bicarbonate buffer (pH 9.6) for overnight incubation at 4 °C. The plate was then blocked with 3% skim milk for 2 h at RT and washed with PBS-T (PBS containing 0.05% Tween 20) three times. Next, 1000-fold diluted serum was added to each well and incubated for 2 h at RT. After washing three times, horseradish peroxidase (HRP)-conjugated goat anti-mouse IgGAM (peroxidase-goat-anti-mouse IgG + A + M; Invitrogen, USA) was added to each well as a secondary antibody and incubated for 1 h at RT. The plate was washed again five times and developed with substrate solution (3,3',5,5'-tetramethylbenzidine, TMB; Invitrogen). The enzyme reaction was terminated by adding 2 N H₂SO₄ solution. The absorbance was measured at 450 nm using a microplate reader (Molecular Devices, USA). To identify the immunoglobulin isotypes in sera, IgG1 or IgG2a (Invitrogen) was used as the secondary antibody. The plate was again washed five times and developed with TMB solution, followed by the addition of 2 N H₂SO₄ solution. The absorbance was measured at 450 nm using a microplate reader (Molecular Devices).

2.5. Cytokine production assay

Splenocytes were harvested from mice which immunized OVA alone (ovalbumin, Sigma, ST USA), OVA+GS-P, OVA+FCA, OVA+FIA or OVA+FIA+GS-P for 2 weeks or 5 weeks, and splenocytes (2 × 10⁶ cells/well, 24-well plate) were cultured with or without OVA (5 µg/ml) in plates at 37 °C in 5% CO₂ incubator. After 72 h, the supernatants collected by centrifugation at 1000 rpm for 5 min. The level of cytokines (IL-2, IFN-γ, GM-CSF, IL-10) in the supernatant were evaluated by indicated ELISA kit (BD Bioscience, USA) according to the manufacture's guideline. The concentration of mouse IgE in serum protein was determined using ELISA kit (BD Biosciences, CA, USA) according to the recommended protocol.

2.6. Lymphocyte proliferation

Splenocytes (5 × 10⁵ cells/well, 96-well plate) were harvested from mice immunized for 5 weeks and co-incubated with or without OVA (100 µL/well) for 72 h. To evaluate splenocyte proliferation, 20 µL of EZ-cytox solution was added to each well and incubated for 3 h. The absorbance was measured at 450 nm using a microplate reader (Molecular Devices Co., CA, USA).

2.7. Statistical analysis

Statistical analysis was performed using Student's *t*-test, with *P* < 0.05 determined to be significant. Results are expressed as the mean ± standard deviation of triplicate experiments.

3. Results and discussion

3.1. Properties of polysaccharides purified from ginseng leaves

In general, hot-water extracts contain both high- and low-molecular-weight materials. Therefore, to remove low-molecular-weight materials, the GS-W fraction was double-refined using Diaion HP-20 (GS-C1 fraction) and Diaion PA312 (GS-C2 fraction) columns. Subsequently, the GS-C2 fraction was further refined by EtOH precipitation and dialysis and was then lyophilized, resulting in a purified GS-P fraction (yield of GS-P, 0.48% w/w). Our previous study indicated that the polysaccharides isolated from *P. ginseng* comprised 15 kinds of monosaccharides, including rhamnose,

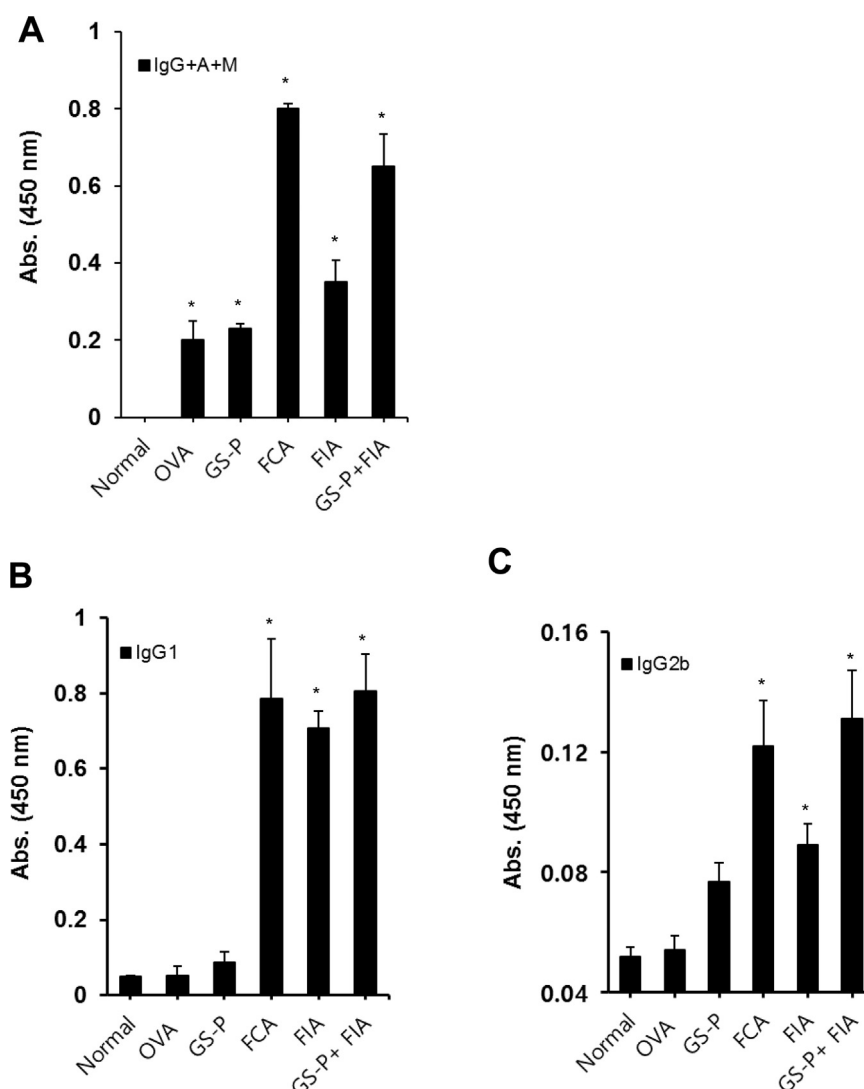


Fig. 1. Effect of GS-P on induction of OVA-specific antibody response. Balb/c mice (n = 3) were immunized subcutaneously twice every 2 weeks with indicated immunogens. IgG + A + M, IgG1, and IgG2b of OVA-specific antibodies were determined with ELISA using 1000-fold diluted mouse sera obtained 5 weeks after primary immunization.

fucose, arabinose, xylose, apiose (Api), mannose, galactose, glucose, 2-methylfucose (2MeFuc), 2-methylxylose (2-MeXyl), aceric acid (AceA), 2-keto-3-deoxy-D-manno-2-octulosonic acid (Dha), 2-keto-3-deoxy-D-lyxo-2-heptulosaric acid (Kdo), galacturonic acid, and glucuronic acid. Among these are six unusual sugars, namely 2-MeFuc, 2-MeXyl, Api, AceA, Kdo, and Dha, which have been identified as components of rhamnogalacturonan (RG)-II-containing polysaccharides of pectic substance isolated from plant cell walls [14–16]. Some studies already reported that biological activity of pectic substances resulted from rhamnogalacturonan-I and -II regions, not homogalacturonan, a linear chain of 1,4-linked α -D-galacturonic acid residues [17,18].

3.2. Effect of GS-P on induction of OVA-specific antibody response

Adjuvants bind antigens by adsorption or entrapment in aluminum hydroxide or a water-in-oil emulsion, and antigens are then released slowly from the vaccination spot, which is critical for adjuvant activity [1,2]. FCA is one of the most popular experimental adjuvants, containing heat-killed and dried *Mycobacterium tuberculosis* [1]. The bacterial components of FCA induce activation of the immune system non-specifically, and the oil components (FIA) entrap antigens in a water-in-oil emulsion, providing the chance

to encounter antigen-presenting cells (APCs) slowly. Therefore, we examined the efficacy of GS-P on enhancement of adjuvant activity. As shown in Fig. 1A, serum antibody production levels were significantly enhanced in the OVA alone (OVA), OVA + GS-P (GS-P), OVA + FCA (FCA), OVA + FIA (FIA), and OVA + GS-P + FIA (GS-P + FIA) groups compared with that in the normal group. Next, the FCA and FIA + GS-P groups showed significantly higher antibody production than the FIA group. FCA is a well-known adjuvant, used as a positive control in our experiments. There were no significant differences in antibody production between mice immunized with FCA and those immunized with FIA + GS-P. This indicates that GS-P might be a substitute for *M. tuberculosis* in FCA. *M. tuberculosis* has been shown to variably interfere with the generation and function of APCs, affecting the cytokine-inducing activity of APC cells such as dendritic cells and macrophages [19]. Indeed, immunization with GS-P induced the stimulation of various cytokines, such as TNF- α and IL-12, by T cells from mouse peritoneal exudate macrophages or dendritic-like cells (data not shown). Therefore, we speculated that one of the reasons for the enhanced antibody production following immunization with GS-P + FIA compared to FIA was the cytokine-producing activity of GS-P. Furthermore, there was no weight loss in mice during experiments.

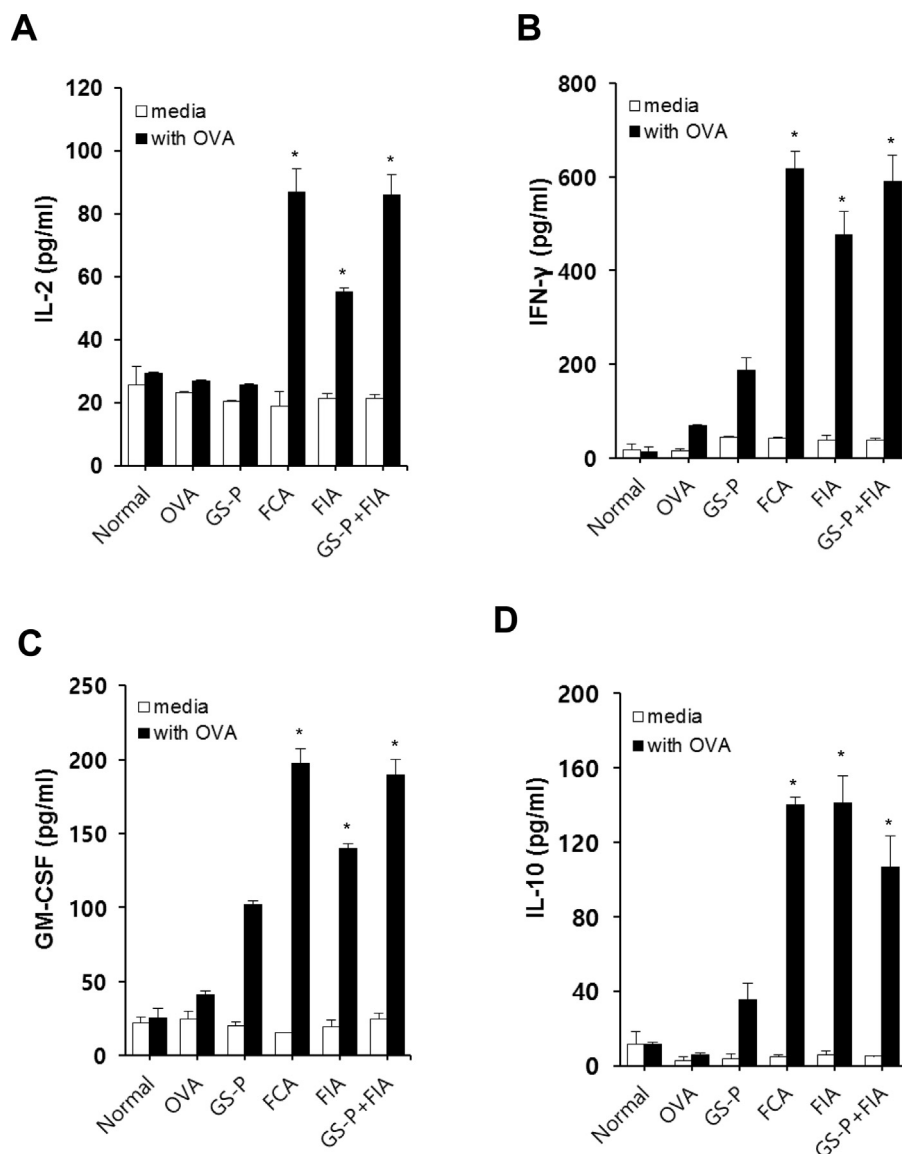


Fig. 2. Effect of GS-P on induction of OVA-specific cytokine production. Splenocytes from mice immunized with the indicated components using the same method described in Fig. 1 were harvested and incubated with OVA (5 μ g/ml) in 24-well plates for 3 days. Levels of IL-2, IFN- γ , GM-CSF, and IL-10 in culture supernatant were measured using ELISA kits.

3.3. Effect of GS-P on changes in OVA-specific antibody subclasses

The production of immunoglobulins (IgG1 and IgG2b) against OVA was measured by ELISA using subclass-specific antibodies (Fig. 1B and C). The FCA, FIA, and GS-P + FIA groups exhibited significantly enhanced OVA-specific serum IgG1 levels compared with those of the OVA or GS-P groups. In contrast, strong increases in IgG2b antibody levels were observed in the FCA and GS-P + FIA groups and a moderate increase was observed in the FIA group. The highest efficacy in anti-OVA IgG2b production was exhibited by FIA + GS-P, followed by FCA, FIA, GS-P, and OVA alone. The GS-P + FIA and FIA groups also exhibited a significant difference compared with levels in control serum. These results suggest that GS-P is effective on Th1 and Th2 cells, associated with an increase in the production of IgG1 and IgG2b [20,21].

3.4. Effect of GS-P on induction of OVA-specific cytokines

In the humoral immune response involving helper T cells, IFN- γ produced by Th1 or IL-4 produced by Th2 regulates the effec-

tor functions of B cells; B lymphocytes to switch to specific Ig classes from IgM [22]. For example, IFN- γ help B cells to produce IgG2-type antibodies, whereas IL-4 differentiate and activate B cells to produce IgG1 and IgE [23–26]. Therefore, we examined the involvement of T lymphocytes in the GS-P-induced increase in the OVA-specific antibody response by assessing the production of antigen-specific Th1-type (IL-2, GM-CSF, and IFN- γ) and Th2-type cytokines (IL-4, IL-6, and IL-10). As shown in Fig. 2, both the FCA- and FIA-immunized groups produced Th1 cytokines such as IL-2, IFN- γ , and GM-CSF (Fig. 2A–C) and Th2 cytokines such as IL-10 (Fig. 2D). There was no cytokine production in the absence of OVA in cultured splenocytes, indicate that the detected cytokines were OVA-specific.

3.5. Effect of GS-P on T lymphocyte proliferation in response to OVA

As shown in Fig. 3, splenocytes harvested from mice immunized with FCA as well as those immunized with GS-P + FIA exhibited much stronger proliferation than those harvested from the FIA

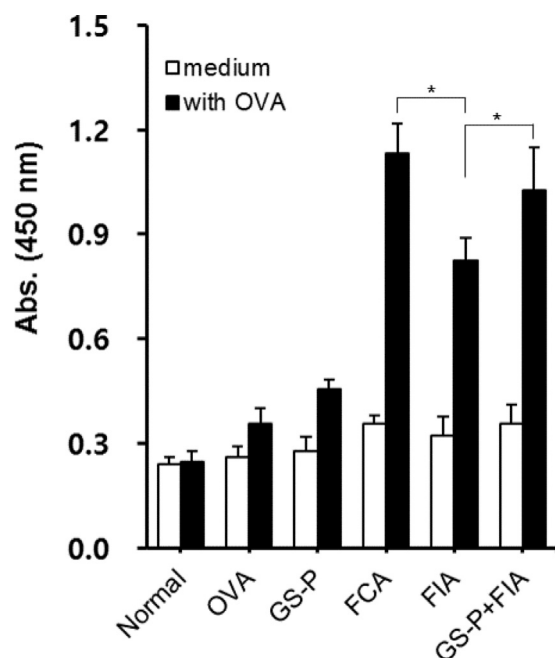


Fig. 3. Effect of GS-P on T lymphocyte proliferation in response to OVA. Splenocytes from mice immunized with the indicated components using the same method described in Fig. 1 were cultured (1×10^6 /well, 96-well plate) with OVA for 3 days. OVA-specific T lymphocyte proliferation was measured by the EZ-cytox method.

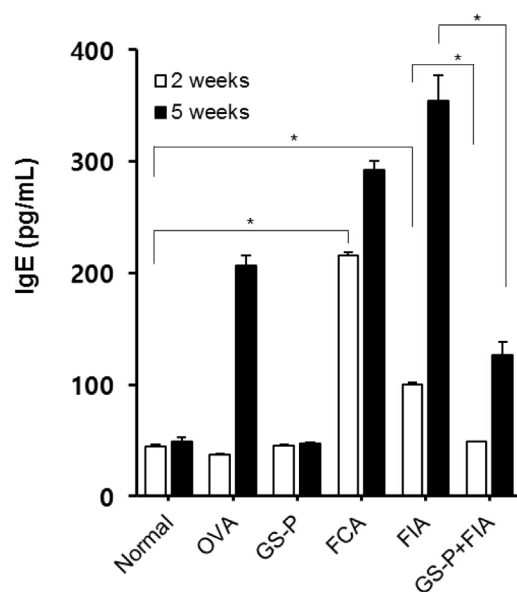


Fig. 4. Determination of IgE levels in mouse serum. Balb/c mice ($n = 3$) were immunized subcutaneously twice every 2 weeks with indicated immunogens. Mouse sera were collected at 2 or 5 weeks after primary immunization. Levels of total IgE were determined with an ELISA kit using 20-fold diluted mouse sera.

group. This suggests that the cellular immune response induction was strongest in the FCA group, followed by the GS-P + FIA and FIA groups, which correlates with the Th1 cytokine profiles and IgG2b antibody production activities (Figs. 1 and 2).

3.6. Effect of GS-P on production of IgE in serum

Next, we evaluated the effect of GS-P on total serum IgE levels in OVA-immunized mice. OVA, which was used in this experiment as an antigen, is a known allergen. As shown in Fig. 4, IgE antibody levels significantly increased beginning at week 2 after

the primary immunization in mice immunized with FCA and FIA. However, the GS-P group did not exhibit an increase in serum IgE levels, and GS-P + FIA immunization decreased the production of IgE compared with levels induced by FCA or FIA. At 5 weeks after immunization, IgE was strongly detected in the OVA-, FCA-, and FIA-immunized groups, but GS-P treatment significantly inhibited FIA-induced IgE production in mouse serum. These results demonstrate that a different antibody response was induced against the same OVA antigen through the use of GS-P. Some studies have documented that the use of liposome or FCA as an adjuvant results in IgE-selective unresponsiveness, which occurs when the immune response is polarized toward the Th1 type [24,25]. However, it has also been reported that the regulation of IgE production may not be due to polarization toward Th1 or Th2 cell subsets [26–28]. Indeed, Taneichi et al. reported that an alternative mechanism not involving T cells exists to regulate the production of IgE [24]. Therefore, the underlying mechanism requires intensive investigation. Consequently, the combination of GS-P and FIA significantly suppress the production of IgE in response to OVA. Taken together, our results demonstrate that the polysaccharides isolated from the leaves of ginseng are a potent adjuvant, enhancing OVA-specific humoral and cellular immune responses. Because GS-P also substantially diminishes the production of IgE, it may have potential use as an adjuvant constituent.

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References

- [1] F.M. Audibert, L.D. Lise, Adjuvants: current status, clinical perspectives and future prospects, *Immunol. Today* 14 (1993) 281–284.
- [2] R.K. Gupta, G.R. Siber, djuvant for human vaccines current status, problems and future prospects, *Vaccine* 13 (1995) 1263–1276.
- [3] S. Helms, Cancer prevention and therapeutics: Panax ginseng, *Altern. Med. Rev.* 9 (2004) 259–274.
- [4] C.X. Liu, P.G. Xiao, Recent advances on ginseng research in China, *J. Ethnopharmacol.* 36 (1992) 27–38.
- [5] C. Wakabayashi, H. Hasegawa, J. Murata, I. Saiki, In vivo antimetastatic action of ginseng protopanaxadiol saponins is based on their intestinal bacterial metabolites after oral administration, *Oncol. Res.* 9 (1997) 411–417.
- [6] M. Mochizuki, Y.C. Yoo, K. Matsuzawa, K. Sato, I. Saiki, S. Tonooka, K. Samukawa, I. Azuma, Inhibitory effect of tumor metastasis in mice by saponins, ginsenoside-Rb2, 20(R)- and 20(S)-ginsenoside-Rg3, of red ginseng, *Biol. Pharm. Bull.* 18 (1995) 1197–1202.
- [7] K.H. Kim, Y.S. Lee, I.S. Jung, S.Y. Park, H.Y. Chung, I.R. Lee, Y.S. Yun, Acidic polysaccharide from Panax ginseng, ginsan, induces Th1 cell and macrophage cytokines and generates LAK cells in synergy with rIL-2, *Planta Med.* 64 (1998) 105–110.
- [8] H.X. Sun, Y.P. Ye, H.J. Pan, Y.J. Pan, Adjuvant effect of Panax notoginseng saponins on the immune responses to ovalbumin in mice, *Vaccine* 22 (2004) 3882–3889.
- [9] S. Hu, C. Concha, F. Lin, K. Persson Waller, Adjuvant effect of ginseng extracts on the immune responses to immunisation against *Staphylococcus aureus* in dairy cattle, *Vet. Immunol. Immunopathol.* 91 (2003) 29–37.
- [10] H.J. Shin, Y.S. Kim, Y.S. Kwak, Y.B. Song, Y.S. Kim, J.D. Park, Enhancement of antitumor effects of paclitaxel (taxol) in combination with red ginseng acidic polysaccharide (RGAP), *Planta Med.* 70 (2004) 1033–1038.
- [11] E. Rivera, S. Hu, C. Concha, Ginseng and aluminium hydroxide act synergistically as vaccine adjuvants, *Vaccine* 21 (2003) 1149–1157.
- [12] Q.P. Gao, H. Kiyohara, J.C. Cyong, H. Yamada, Chemical properties and anti-complementary activities of heteroglycans from the leaves of Panax ginseng, *Planta Med.* 57 (1991) 132–136.
- [13] X.B. Sun, T. Matsumoto, H. Yamada, Purification of an anti-ulcer polysaccharide from the leaves of Panax ginseng, *Planta Med.* 58 (5) (1992) 445–448.
- [14] K.S. Shin, H. Kiyohara, T. Matsumoto, H. Yamada, Rhamnogalacturonan II from the leaves of Panax ginseng CA Meyer as a macrophage Fc receptor expression-enhancing polysaccharide, *Carbohydr. Res.* 300 (1997) 239–249.
- [15] M.S. Shin, S.H. Hwang, T.J. Yoon, K.S. Shin, Polysaccharides from ginseng leaves inhibit tumor metastasis via macrophage and NK cell activation, *Int. J. Biol. Macromol.* 103 (2017) 1327–1333.

- [16] P. Albersheim, J. An, G. Freshour, M.S. Fuller, R. Guillen, K.S. Ham, M.V. Williams, Structure and function studies of plant cell wall polysaccharides, *Biochem. Soc. Trans.* 22 (2) (1994) 374–378.
- [17] R. Srivastava, D.K. Kulshreshtha, Bioactive polysaccharides from plants, *Phytochemistry* 28 (1989) 2877–2883.
- [18] H. Yamada, Chemical and pharmacological studies on efficacy of Japanese and Chinese herbal medicines, *Kitasato Arch. Exp. Med.* 65 (1992) 159.
- [19] A.D. Edwards, S.P. Manickasingham, R. Sporri, S.S. Diebold, O. Schulz, A. Sher, T. Kaisho, S. Akira, C. Reis Sousa, Microbial recognition via Toll-like receptor-dependent and –independent pathways determines the cytokine response of murine dendritic cell subsets to CD40 triggering, *J. Immunol.* 169 (2002) 3652–3660.
- [20] A. Sjolander, B. van't Land, K. Lovgren Bengtsson, Iscoms containing purified Quillaja saponins upregulate both Th1-like and Th2-like immune responses, *Cell. Immunol.* 177 (1997) 69–76.
- [21] T.J. Yoon, Y.C. Yoo, T.B. Kang, E. Her, S.H. Kim, K. Kim, I. Azuma, J.B. Kim, Cellular and humoral adjuvant activity of lectins isolated from Korean mistletoe (*Viscum album colaratum*), *Int. Immunopharmacol.* 1 (2001) 881–889.
- [22] Y. Aramaki, H. Suda, S. Tsuchiya, Interferon-gamma inductive effect of liposomes as an immunoadjuvant, *Vaccine* 13 (1995) 1809–1814.
- [23] T.R. Mosmann, S. Sad, The expanding universe of T-cell subsets: Th1, Th2 and more, *Immunol. Today* 17 (1996) 138–146.
- [24] M. Taneichi, S. Naito, H. Kato, Y. Tanaka, M. Mori, Y. Nakano, H. Yamamura, H. Ishida, K. Komuro, T. Uchida, T cell-independent regulation of IgE antibody production induced by surface-linked liposomal antigen, *J. Immunol.* 169 (2002) 4246–4252.
- [25] L. Beck, H.L. Spiegelberg, The polyclonal and antigen-specific IgE and IgG subclass response of mice injected with ovalbumin in alum or complete Freund's adjuvant, *Cell. Immunol.* 123 (1989) 1–8.
- [26] A. Kelso, Th1 and Th2 subsets: paradigms lost? *Immunol. Today* 16 (1995) 374–379.
- [27] G.S. Firestein, W.D. Roeder, J.A. Laxer, K.S. Townsend, C.T. Weaver, J.T. Hom, J. Linton, B.E. Torbett, A.L. Glasebrook, A new murine CD4⁺ T cell subset with an unrestricted cytokine profile, *J. Immunol.* 143 (1989) 518–525.
- [28] V. Arps, S. Sudowe, E. Kolsch, Antigen dose-dependent differences in IgE antibody production are not due to polarization towards Th1 and Th2 cell subsets, *Eur. J. Immunol.* 28 (1998) 681–686.