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Platycodin D is a potent adjuvant of specific cellular and humoral immune responses against recombinant hepatitis B antigen

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ABSTRACT

The ideal adjuvants for hepatitis B vaccines should be capable of eliciting both strong humoral and cellular immune responses, especially Th1 cell and cytotoxic T lymphocyte (CTL) responses. However, Alum used as adjuvants in the hepatitis B vaccines currently commercialized offers limitation in inducing cell-mediated response. Therefore, a less hemolytic saponin platycodin D (PD) from the root of *Platycodon grandiflorum* has been explored for its potential as an alternative adjuvant. In order to compare the adjuvant activity with Alum, antigen-specific cellular and humoral immune responses were evaluated following immunization with a formulation containing hepatitis B surface antigen (HBsAg) adjuvanted with PD and Alum in mice. The Con A-, LPS-, and HBsAg-induced splenocyte proliferation and the serum HBsAg-specific IgG, IgG1, IgG2a, and IgG2b antibody titers in the HBsAg-immunized mice were significantly enhanced by PD ($P<0.05$, $P<0.01$ or $P<0.001$). PD also significantly promoted the production of Th1 (IL-2 and IFN-γ) and Th2 (IL-10) cytokines and up-regulated the mRNA expression of Th1 cytokines (IL-2 and IFN-γ) in splenocytes from the mice immunized with HBsAg ($P<0.001$). Besides, PD remarkably increased the killing activities of natural killer (NK) cells and CTLs from splenocytes in the HBsAg-immunized mice ($P<0.001$), which may have important implications for vaccination against hepatitis B virus. The results indicated that PD has strong potential to increase both cellular and humoral immune responses and elicit a balanced Th1/Th2 response against HBsAg, and that PD may be the candidates as adjuvants for use in prophylactic and therapeutic hepatitis B vaccine.

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1. Introduction

Hepatitis B virus (HBV) is a common virus. Two billion people have been infected with HBV, and 360 million have chronic infection worldwide [1]. Once infected, 5–10% adults and 25–90% children become chronic virus carriers [2]. About 25% of HBV carriers die from chronic active hepatitis, cirrhosis, or primary liver cancer [3]. Chronic infection with HBV has become a major health problem worldwide [4]. No treatment is currently available for acute Hepatitis B, and immunization with vaccine is the effective means of preventing HBV infection and its consequences [1]. Hepatitis B surface antigen (HBsAg) adsorbed on Alum is the commonly used vaccine against HBV, and has been used for many years to induce protection against HBV infection. Though recognized as an effective vaccine, nearly 10% of vaccinees with HBsAg fail to develop protective levels of anti-HBsAg antibodies [5]. Thus, approaches to improve vaccination efficacy are actively sought.

It is well accepted that strong antibody responses are important for clearing the HBV and protection against HBV infection. There are some increasing evidences showing that the Th1 cell and CTL response to HBV and the associated antiviral cytokines (IFN-γ, TNF-α and IL-2) developed may play a key role in virus resolution during natural infection [6,7]. Optimization of vaccine effectiveness could be achieved by the use of appropriate adjuvants which could enhance vaccine coverage of non-responders [8]. Currently, aluminum compounds (Alum) are the only vaccine adjuvants licensed by the Food and Drug Administration (FDA) for use in humans, and used as adjuvants in the hepatitis B vaccines currently commercialized. While Alum is safe, it is a relatively weak adjuvant particularly when used with subunit antigens. Moreover, the Alum adjuvant used in the hepatitis B vaccine is a mild Th2 adjuvant that can effectively enhance IgG1 antibody responses, but it is rarely associated with Th1 type immune responses [9]. Furthermore, Alum is poor at stimulating cell-mediated immune responses, and may actively block activation and differentiation of $CD8^+$ CTLs follow-

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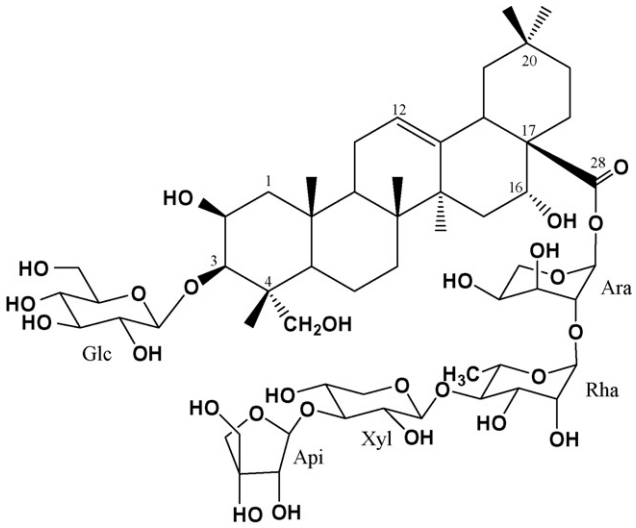

Fig. 1. Chemical structure of platycodin D (PD, $C_{57}H_{92}O_{28}$, MW: 1224.5854) from the roots of *P. grandiflorum*. Its structure was elucidated on the basis of chemical and spectral evidences including mass spectrometry and nuclear magnetic resonance spectroscopy. Api, β-D-apiofuranosyl; Ara, α-L-arabinopyranosyl; Glc, β-D-glucopyranosyl; Rha, α-L-rhamnopyranosyl; Xyl, β-D-xylopyranosyl.

ing immunization with HBsAg [10]. In addition, Alum results in occasional local reactions at the site of injection and augmentation of IgE responses. The condition can hardly satisfy the needs of development of vaccines [11]. The ideal adjuvants for hepatitis B vaccines should be capable of eliciting both strong humoral and cellular immune responses, especially Th1 cell and CTL responses [12].

Platycodin D (PD) is a saponin isolated from the root of *Platycodon grandiflorum*, has two unbranched sugar chains attached to the carbon C-3 and C-28 in the aglycones, with each chain being composed of one and four monosaccharide residues, and without acyl domain in its molecule (Fig. 1). Compared with QS-21, PD is less hemolytic and very stable in aqueous phase. Meanwhile, its plant resources are very abundant and the preparation is convenient. PD has also been shown to significantly enhance a specific antibody and cellular response against OVA in mice and simultaneously elicit a Th1 and Th2 immune response by regulating gene expression of Th1/Th2 cytokines and transcription factors [13]. This study is aimed to use PD as an adjuvant in the hepatitis B vaccines so that it can provides a simple and safe alternative for improving the potency of vaccines and replacing Alum salts in vaccines to induce cell-mediated immune response.

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

2.2. Materials

The HBsAg used was the recombinant product and purified from *Saccharomyces cerevisiae* at Shenzhen Kangtai Biological Products Co., Ltd., Guangzhou, China. 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. Trizol was from Invitrogen, USA; revertAid™ M-MuLV reverse transcriptase was from Fermentas, USA; diethylpyrocarbonate (DEPC) and ribonuclease inhibitor were from Biobasic, Canada; Oligo$(dT)_{18}$ were from Sangon, China; SYBR® Premix Ex Taq™ (Perfect Real Time) PCR kit was purchased from TaKaRa; 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.

2.3. Cell lines

Mouse lymphadenoma YAC-1 cell lines, sensitive to natural killer (NK) cells, were purchase from Institute of Cell Biology, Chinese Academy Sciences. Mouse hepatoma H22 cell lines were provided by Zhejiang Academy of Medical Sciences. Yac-1 and H22 cells were used as target cells for NK cells and CTL activity assay, respectively. They was 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.

2.4. Extraction, isolation and identification of platycodin D

Platycodin D (PD, Fig. 1) was isolated from the root of *Platycodon grandiflorum* according to a previously published method [13], and was identified on basis of chemical and spectroscopic evidence (IR, MS, and NMR experiments including ^{1}H- and ^{13}C NMR, ^{1}H,^{1}H-COSY, HMQC, and HMBC). The purity of PD was determined to be 99.09% by HPLC on a Water 600E HPLC instrument with a Symmetry® C18 column (250 mm × 4.6 mm i.d.; 5 μm particle size), a Waters 2996 PDA detector, eluting with MeOH/H_2O 52:48 (t_R 21.52 min).

A stock compound solution with a concentration of 4 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). The endotoxin level in the stock solution was less than 0.5 endotoxin units (EU)/ml.

2.5. Immunization

Six-week-old female ICR mice were divided into six groups, each consisting of five mice. Animals were immunized subcutaneously with HBsAg 2 μg alone or with HBsAg 2 μg dissolved in 0.2 ml phosphate buffered solution (PBS) containing Alum (50 μg), or Platycodin D (50, 75, or 100 μg) on Day 1. PBS-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 HBsAg-specific antibody and proliferation, NK cell and CTL activity, and cytokine assay.

2.6. Splenocyte proliferation assay

Spleen collected from the HBsAg-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 ($380 \times g$ 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 hemocytometer by trypan blue dye exclusion technique. Cell viability exceeded 95%. Splenocytes were seeded into three to four wells of a 96-well flat-bottom microtiter plate (Nunc) at 5×10^6 cells/ml in 100 μl complete medium, thereafter Con A (final concentration 5 μg/ml), LPS (final concentration 10 μg/ml), HBsAg (final concentration 4 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 (for Con A and LPS) or 68 h (for HBsAg), 50 μl of MTT solution (2 mg/ml) was added to each well and incubated for further 4 h. The plates were centrifuged ($1400 \times g$, 5 min) and the untransformed MTT was removed carefully by pipetting 150 μl of a DMSO (Sigma, USA) working solution (192 μl DMSO with 8 μl 1N HCl) was added to each well, and the absorbance was evaluated in an ELISA reader at 570 nm after 15 min. The stimulation index (SI) was calculated based on the following formula: SI = the absorbance value for mitogen-cultures divided by the absorbance value for non-stimulated cultures.

2.7. Measurement of HBsAg-specific antibody

HBsAg-specific IgG, IgG1, IgG2a, and IgG2b antibodies in sera were detected in individual serum samples by an indirect ELISA. In brief, microtiter plate wells were coated with 100 μl HBsAg solution (2 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 1:16,000, IgG2a peroxidase conjugate 1:8000, and IgG2b peroxidase conjugate 1:8000 with 0.5% FCS/PBS were added to each plate. The plates were further incubated for 2 h at 37 °C. After washing, the peroxidase activity was assayed as following: 100 μl of substrate solution (10 mg of *O*-phenylenediamine and 37.5 μl of 30% H_2O_2 in 25 ml of 0.1 M citrate–phosphate buffer, pH 5.0) was added to each well. The plate was incubated for 10 min at 37 °C, and enzyme reaction was terminated by adding 50 μl/well 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 NK cell activity from spleen was determined using a Cytotox 96R Non-Radioactive Cytotoxicity Assay Kit (Promega). YAC-1 cells were used as target cells and seeded in 96-well U-bottom microtiter plate at 4×10^4 cells/well in RPMI-1640. Splenocytes prepared as described above were used as the effector cells and were added at 2×10^6 cells/well to give E/T ratio 50:1. Each test was repeated four wells. The plate was centrifuged at $250 \times g$ for 1 min to ensure effecter and target cell contact. After 4 h incubation in a humidified 37 °C, 5% CO_2, the plate was again centrifuged at $250 \times g$ for 4 min. The supernatant from each well (100 μl) was transferred into the corresponding wells of a 96 flat-bottom microtiter plate. Lactate dehydrogenase (LDH) activity was measured in the supernatants by addition of the enzyme substrate and absorbance recording at 490 nm. Three kinds of control measurements were performed: an effector cell spontaneous release, a target cell spontaneous release and a target cell maximum release. NK cell activity was calculated as following equation: NK cell activity (%) = $(A_S - A_E - An)/(Am - An) \times 100$, where A_S, absorbance value of test samples; A_E, absorbance value of effector cells control; An, absorbance value of target cells incubated with medium; Am, absorbance value of target cells incubated with 1% of NP-40.

2.9. Assays of HBsAg-specific cytotoxic T lymphocyte (CTL) response

The antigen-specific CTL response was evaluated as previously described [14] with some modifications. Briefly, splenocytes prepared as described above were used as the effector cells and resuspended in two aliquots of 1×10^7 cells/ml each. The cells of the first aliquot (1 ml) were seeded in a 24-well plate and primed with HBsAg (final concentration 4 μg/ml), and the second aliquot was incubated in medium only. After 7 d incubation in a humidified 37 °C, 5% CO_2, the plate was again centrifuged at $380 \times g$ at 4 °C for 10 min. The pelleted cells from the first and second aliquots were washed three times with PBS and resuspended in complete medium at a final concentration of 1×10^7 cells/ml as Ag-stimulated effector cells (Es) and un-pulsed effector cells (nE). H22 cells were used as target cells and cultured with or without HBsAg for 24 h as described above. After centrifugation, the pelleted cells were washed three times with PBS and resuspended to 2×10^5 cells/ml with complete medium as Ag-stimulated target cells (Ts) and un-pulsed target cells (nT). Effector cells and target cells were seeded into four wells of a 96-well U-bottom microtiter plate in 100 μl complete medium to give E/T ratio 50:1 as following group: A, Es + Ts; B, Es + nT; C, nE + Ts; D, nE + nT. Three kinds of control measurements were performed: an effector cell spontaneous release, a target cell spontaneous release and a target cell maximum release. The plate was centrifuged at $250 \times g$ for 1 min to ensure effecter and target cell contact. After 4 h incubation in a humidified 37 °C, 5% CO_2, the plate was again centrifuged at $250 \times g$ for 4 min. LDH activity in the supernatants was measured using a Cytotox 96R Non-Radioactive Cytotoxicity Assay Kit (Promega). To determine the percentage of target cells killed, the following equation was used: % lysis = $(A_S - A_E - An)/(Am - An) \times 100$, where A_S, absorbance value of test samples; A_E, absorbance value of effector cells control; An, absorbance value of target cells incubated with medium; Am, absorbance value of target cells incubated with 1% of NP-40. Specific lysis expressed as % was calculated as: $\%CTL_{specific} = (\%\ lysis_A - \%\ lysis_B) - (\%\ lysis_C - \%\ lysis_D)$.

2.10. 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 HBsAg (final concentration 4 μg/ml) in 24-well culture plates at 37 °C in 5% CO_2. After 48 h, the plate was centrifuged at $1400 \times g$ 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 (IFN-γ for 2 h). Plates were washed and a detecting antibody was added to each well. Plates were incubated at 37 °C for 1 hour before addition of avidin–biotin–peroxidase com-

Table 1
Sequences of primer used for real-time quantitative RT-PCR.

Gene	Primer sequence	Product size/bp	Acc no.
GAPDH	5'-AAATGGTGAAGGTCGGTGTG-3' 5'-TGAAGGGGTCGTTGATGG-3'	108	NM_001001303
IL-2	5'-GCACCCACTTCAAGCTCCA-3' 5'-AAATTTGAAGGTGAGCATCCTG-3'	174	NM_008366
IFN-γ	5'-CGGCACAGTCATTGAAAGCCTA-3' 5'-GTTGCTGATGGCCTGATTGTC-3'	199	NM_008337

GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

plex (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.

2.11. Real-time RT-PCR for cytokine gene expression

Splenocytes from the immunized mice prepared as described before were seeded into a 24-well flat-bottom microtiter plate (Nunc) at 5×10^6 cell/ml in 1 ml complete medium, thereafter HBsAg (final concentration 4 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 24 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 0.5 ml of Trizol reagent and the total RNA was isolated according to the manufacture'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 11 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 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.

All primers and probes used in this study were designed according with Primer Express software (Applied Biosystems) and synthesized at TAKARA Co. PCR primer and fluorogenic probe sequences and references for all target genes are shown in Table 1. The amplification of each primer pair was optimized by testing at different temperatures (55 and 60 °C) and primer concentration (100, 300, 600 nM), using a fixed quantity of target template. Optimal conditions provided the lowest Ct and highest Tm. PCR amplification was undertaken for plain SYBR Green I detection using the LightCycler System (Roche Diagnostics GmbH, Mannheim, Germany). Each sample was assayed for cytokine expression and for 18S rRNA as an endogenous control in the same tube in 20 μl RT-PCR mixture. SYBR® Premix Ex Taq™ (Perfect Real Time) PCR kit was purchased from TaKaRa. Two microliters of cDNA was used as the template. The reaction was carried out in a 20 μl final reaction volume containing 10 μl of kit-supplied SYBR® PCR Master mix (including HotStart Ex Taq HS DNA polymerase, reaction buffer, dNTP mix and SYBR Green I), 2 μl DNA template, each forward and reverse primer (0.4 μmol/l) and distilled water. Prior to cycling, the glass capillaries were sealed, placed into the LC sample carousel, and centrifuged at 400 × *g* for 10 s in the LC carousel centrifuge (Eppendorf, Hamburg, Germany). The reaction was carried out using the following conditions: one cycle of 95 °C for 10 s, 60 cycles of 95 °C for 5 s/60 °C for 20 s with a single fluorescence detection point at the end of the annealing/extension segment. After this, one cycle of melting curve from 55 to 95 °C by a transition rate of 0.1 °C with continuous detection of fluorescence, was performed. The threshold cycle (Ct), i.e. the cycle number at which the amount of amplified gene of interest reached a fixed threshold, was determined subsequently. Relative quantization of IL-2 and IFN-γ mRNA expression was calculated by the comparative Ct method. The relative quantization value of target, normalized to an endogenous control GAPDH gene and relative to a calibrator, is expressed as $2^{-\Delta\Delta Ct}$ (fold), where ΔCt = Ct of target gene (IL-2 or IFN-γ) − Ct of endogenous control gene (GAPDH), and ΔΔCt = ΔCt of samples for target gene − ΔCt of the calibrator for the target gene.

2.12. 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. Effect of PD on splenocyte proliferation in HBsAg-immunized mice

The effects of PD on mitogen- and HBsAg-stimulated splenocyte proliferation in mice immunized with HBsAg are shown in Fig. 2. Con A-stimulated splenocyte proliferation in the mice immunized with PD at three doses was significantly higher than that in the HBsAg group and HBsAg/Alum group ($P < 0.05$ or $P < 0.01$). Splenocytes isolated from mice immunized with HBsAg and PD and stimulated by LPS and HBsAg showed a greater proliferative response than that observed for the mice immunized with HBsAg alone and HBsAg/Alum ($P < 0.01$ or $P < 0.001$). However, no significant differences ($P > 0.05$) were observed between the HBsAg group and HBsAg/Alum group. These data indicate that PD is more effective than Alum in inducing strong activation potential of T and B cells in HBsAg-immunized mice.

3.2. Effect of PD on the HBsAg-specific serum antibody response

To investigate the effect of PD on the induction of humoral immune responses against HBsAg in mice, groups of mice were immunized two times by *s.c.* routes. The HBsAg-specific IgG,

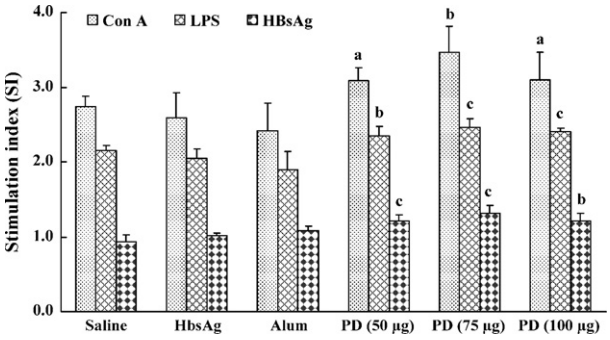

Fig. 2. Effects of platycodin D (PD) on mitogen- and HBsAg-stimulated splenocyte proliferation in mice immunized with HBsAg. Splenocyte proliferation was measured by the MTT method as described in the text, and shown as a stimulation index. The values are presented as means ± S.E. ($n = 5$). Significant differences with HBsAg alone and HBsAg/Alum groups were designated as $^aP < 0.05$, $^bP < 0.01$, and $^cP < 0.001$.

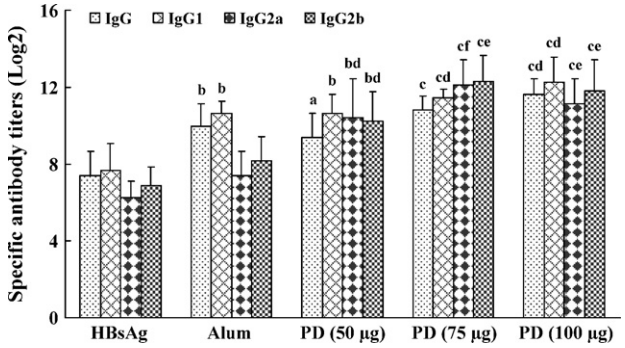

Fig. 3. Effect of platycodin D (PD) on HBsAg-specific IgG, IgG1, IgG2a, and IgG2b antibody in HBsAg-immunized mice. Sera were collected 2 weeks after the last immunization, and HBsAg-specific IgG, IgG1, IgG2a, and IgG2b antibodies in the sera were measured by an indirect ELISA as described in the text. The values are presented as means ± S.E. ($n = 5$). Significant differences with HBsAg alone group were designated as $^{a}P < 0.05$, $^{b}P < 0.01$, and $^{c}P < 0.001$; those with HBsAg/Alum group as $^{d}P < 0.05$, $^{e}P < 0.01$, and $^{f}P < 0.001$.

IgG1, IgG2a, and IgG2b antibody titers in the serum were measured 2 weeks after the last immunization using ELISA and the results were shown in Fig. 3. HBsAg alone induced low levels of HBsAg-specific IgG, IgG1, IgG2a, and IgG2b antibody. Alum and PD significantly enhanced the serum HBsAg-specific IgG and IgG1 titers in HBsAg-immunized mice ($P < 0.05$, $P < 0.01$, or $P < 0.001$). However, the combination of HBsAg with PD especially at dose of 100 μg produced higher serum total IgG and IgG1 titers compared with Alum and HBsAg combinations. Significant enhancements in HBsAg-specific serum IgG2a and IgG2b titers were observed in PD-immunized mice compared with HBsAg alone group ($P < 0.01$ or $P < 0.001$). Moreover, IgG2a and IgG2b antibody titers in the mice immunized with PD were higher than those in the Alum-treated mice. There were, however, no significant differences ($P > 0.05$) in the serum IgG2a and IgG2b levels between mice groups immunized with HBsAg/Alum and HBsAg alone. The findings indicated that PD significantly enhanced serum HBsAg-specific antibody production in mice immunized with HBsAg. Moreover, antibody titers induced by PD-adjuvanted HBsAg after two injections were still higher than those elicited by Alum-adjuvanted vaccine.

3.3. Effects of PD on NK cell activity in mice immunized with HBsAg

The effects of PD on NK cell activity in mice immunized with HBsAg were shown in Fig. 4. PD significantly enhanced the killing activity of NK cell in the HBsAg-immunized mice at three

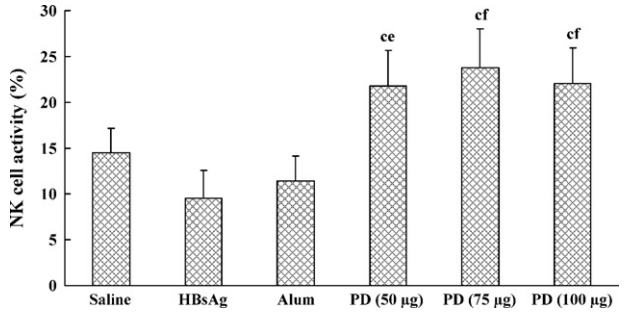

Fig. 4. Effect of platycodin D (PD) on NK cell activity in mice immunized with HBsAg. Splenocytes were prepared 2 weeks after the last immunization, and assayed for NK cell activity by the LDH release method as described in the text. The values are presented as means ± S.E. ($n = 5$). Significant differences with HBsAg alone group were designated as $^{c}P < 0.001$; those with HBsAg/Alum group as $^{e}P < 0.01$ and $^{f}P < 0.001$.

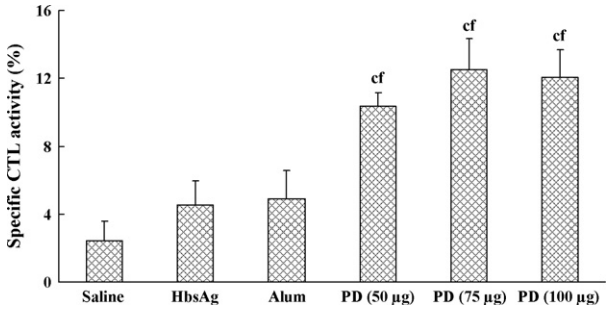

Fig. 5. Effect of platycodin D (PD) on CTL activity in mice immunized with HBsAg. Splenocytes were prepared 2 weeks after the last immunization, and assayed for CTL activity by the LDH release method as described in the text. The values are presented as means ± S.E. ($n = 5$). Significant differences with HBsAg alone and HBsAg/Alum groups were designated as $^{c}P < 0.001$ and $^{f}P < 0.001$, respectively.

doses ($P < 0.001$). There were, however, no significant differences ($P > 0.05$) in the killing activity of NK cell between mice groups immunized with HBsAg/Alum and HBsAg alone. The findings indicated that PD could promote activation of NK cell lytic activity in mice immunized with HBsAg.

3.4. Effects of PD on specific CTL activity in mice immunized with HBsAg

The effects of PD on antigen-specific CTL activity in the HBsAg-immunized mice were shown in Fig. 5. Immunization of mice with HBsAg alone induced the lower specific CTL activity 2 weeks after the secondary immunization. Addition of Alum to HBsAg did not result in further increase in HBsAg-specific CTL activity above those seen with HBsAg alone. In contrast, PD significantly enhanced the specific killing activity of CTL in mice immunized with HBsAg at three doses ($P < 0.001$). The findings indicated that PD could promote specific killing activity of CTL in mice immunized with HBsAg.

3.5. Effect of PD on cytokine secretion by splenocytes from HBsAg-immunized mice

In order to assess effect of PD on Th1 and Th2 cytokines responding to HBsAg, cytokine productions from splenocytes in HBsAg-immunized mice were detected using ELISA. The calibration curves of IL-2, IFN-γ, and IL-10 were constructed with mouse cytokine standards, and their correlation coefficient were all bigger than 0.9980. As shown in Fig. 6, the contents of cytokines IL-2, IFN-γ, and IL-10 in the culture supernatants from HBsAg-stimulated splenocytes of the mice immunized with HBsAg/PD were significantly higher than those in HBsAg control mice ($P < 0.01$), suggesting that PD significantly enhanced the production of the Th1 and Th2 cytokines in HBsAg-immunized mice. However, Alum significantly increased only the IL-10 production the HBsAg-immunized mice ($P < 0.001$).

3.6. Effect of PD on mRNA expression of cytokines in splenocytes from HBsAg-immunized mice

Since PD significantly enhanced IgG2a and IgG2b antibody responses and Th1 cytokine production, we next measured IL-2 and IFN-γ mRNA expression in splenocytes from mice immunized with HBsAg. The splenocytes from the immunized mice were stimulated in vitro with HBsAg, and total RNA were extracted. Real-time quantitative RT-PCR for IL-2 and IFN-γ cytokine mRNA expressions were performed. GAPDH was used as a normalization control for the RT-PCR reactions. As shown in Fig. 7, the IL-2 and IFN-γ mRNA expression in the mice immunized with PD were higher than

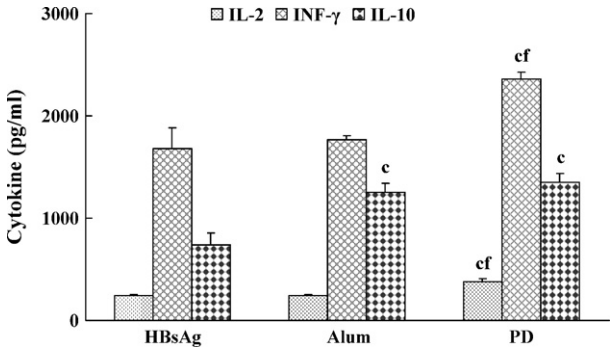

Fig. 6. Effects of platycodin D (PD) on HBsAg-induced cytokine production in splenocytes from the HBsAg-immunized mice. Splenocytes were prepared 2 weeks after the last immunization, and cultured with HBsAg (final concentration 4 μg/ml) for 48 h. The culture supernatants were collected, and the contents of cytokines IL-2, IFN-γ, and IL-10 were determined by ELISA. The values are presented as means ± S.E. ($n = 5$). Significant differences with HBsAg alone and HBsAg/Alum groups were designated as $^{c}P < 0.001$ and $^{f}P < 0.001$, respectively.

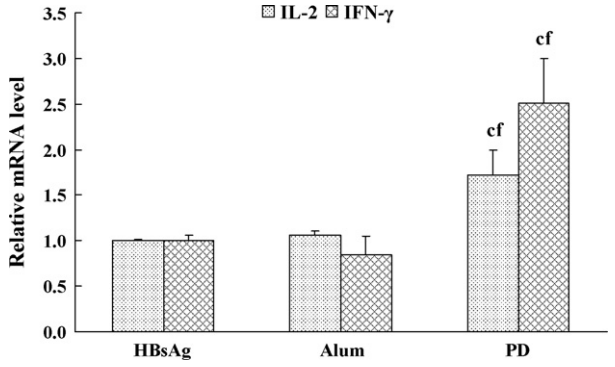

Fig. 7. Effect of platycodin D (PD) on mRNA expression of cytokines IL-2 and IFN-γ in splenocytes from the HBsAg-immunized mice. Splenocytes were prepared 2 weeks after the last immunization, and cultured with HBsAg (final concentration 4 μg/ml) for 24 h. The mRNA expression level of GAPDH, IL-2, and IFN-γ were detected by real-time RT-PCR using specific primers. The relative mRNA expression level are presented as means ± S.E. ($n = 5$). Significant differences with HBsAg alone and HBsAg/Alum groups were designated as $^{c}P < 0.001$ and $^{f}P < 0.001$, respectively.

those in the HBsAg alone and HBsAg/Alum groups ($P < 0.001$). There were, however, no significant differences ($P > 0.05$) in the IL-2 and IFN-γ mRNA expressions between mice groups immunized with HBsAg/Alum and HBsAg alone. Thus, findings further confirmed that PD significantly induced Th1 cytokine secretion by splenocytes from the HBsAg-immunized mice.

4. Discussion

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 [15,16]. 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 [17]. Th1 responses are associated with IFN-γ, IL-2 and IL-12 production and enhance immunoglobulin isotype switching to IgG2a, IgG2b and IgG3. The Th1 response can be correlated with the induction of cell-mediated immunity [18], which has been described as being implicated for the elimination of virus during infection [19,20]. Th2 responses, which control the humoral immune response through the triggering of B cell proliferation and differentiation [21], are characterized by high levels of circulating antibodies, of mainly IgG1 isotype, and the secretion of the cytokines IL-4, IL-5, IL-10 and IL-13. For protective immunity to certain infectious diseases differential induction of Th1 or Th2 responses or both is required.

Alum is used as adjuvants in the hepatitis B vaccines currently commercialized. Alum-adsorbed vaccines have been shown to elicit essentially Th2 immune responses, and to poorly induce cell-mediated immunity. It was reported that a Th2 adjuvant, such as Alum, is not efficacious in seroconversion in this non-responding population, while an adjuvant inducing a balanced Th1/Th2 response against HBsAg was able to overcome non-responsiveness to HBsAg in B10.M mice. Meanwhile, cellular immunity now appears essential to obtain a more efficient response against virus infections by helping achieve total viral elimination. Consequently, it may be desirable for vaccines against viruses to elicit cell-mediated immunity, as well as producing effective antibodies. Superior and universal adjuvants must be developed that work with almost every antigen, induce both cellular and humoral immune responses (a balanced Th1/Th2 response). The extensive studies on plant based adjuvants such as QS21 saponins from plant *Quillaja saponaria* [22], RLJ-NE-299A glycosides from *Picrorhiza kurroa* [23], BOS 2000 from *Boswellia serrata* [24], β-(1→6)-branched β-(1→3) glucohexaose analogue [8] indicate that these are excellent candidates to replace Alum as the adjuvant of choice for many vaccines. Particular advantages offered by plant based proposed adjuvants in inducing cellular in addition to humoral immunity offer excellent safety, tolerability, ease of manufacture and formulation. Thus, adjuvants based on plants have enormous potential for use in vaccines against both pathogens and cancer [25].

Platycodin D is a potential less hemolytic saponin adjuvant isolated from the root of *Platycodon grandiflorum*, and significantly enhanced a specific antibody and cellular response against OVA in mice and simultaneously elicited a Th1 and Th2 immune response [13]. It is proposed as an interesting alternative adjuvant to the Alum, to induce both humoral as well cell-mediated immune response against HBsAg. The current study was undertaken to evaluate the adjuvant potential of PD to enhance the cellular and humoral immune responses and to induce a CTL response in mice against HBsAg in comparison with Alum.

The lymphocyte-mediated immunity plays an important role in combating intracellular microbe infections. 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 PD could significantly promote the Con A-, LPS-, and HBsAg-stimulated splenocyte proliferation in HBsAg-immunized mice. There were, however, no significant differences between the HBsAg group and HBsAg/Alum group. The results indicated that PD could significantly increase the activation potential of T and B cells in HBsAg-immunized mice.

It has been demonstrated that HBsAg-specific antibody plays an important role in preventing HBV infection [26,27]. Therefore, we evaluated the adjuvant activity of PD on the humoral immune responses to HBsAg. We found that addition of PD to HBsAg at the dose of 100 μg resulted in dramatic increases in IgG antibody responses to levels that exceeded those induced by Alum, the "gold-standard" for human vaccines. Evaluation of IgG1 and IgG2a, IgG2b antibody isotypes in response to immunization provide a relative measure of the contribution of Th2 and Th1 humoral immune responses, respectively [28]. In our studies, while antigen alone induced low levels of IgG1, IgG2a, and IgG2b antibodies, the addition of PD to HBsAg at three doses tested resulted in dramatic increases in IgG1, IgG2a, and IgG2b antibody titers. This clearly

demonstrated that PD modulated the quality of immune responses, and elicited a balanced Th1/Th2 immune response to HBsAg in mice as associated sensitively with an enhancement of IgG2a, IgG2b and IgG1 levels [29].

In order to clearly establish that Th cell-derived cytokines were involved in the adjuvant activity of PD, we analyzed the Th1/Th2 cytokine secretion profiles in HBsAg-immunized mice using ELISA. PD 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 HBsAg-immunized mice. High IL-2 secretion correlated with the induction of an antigen-specific cellular proliferative response, while the high level of IFN-γ is consistent with the increase of IgG2a and IgG2b antibodies. Similarly, the mice immunized with Alum and PD had high level of IL-10 and corresponded to the high levels of IgG1 titers in the mice. Together, the HBsAg-specific antibody isotypes and cytokine profiles confirm that the PD promoted a balanced Th1/Th2 type immune response, while Alum was associated with predominantly Th2 type immune responses. In order to further elucidate the mechanism responsible for the changes in the amounts of Th1 cytokines, we utilized real-time RT-PCR to analysis the mRNA expression of the archetypal Th1 cytokines IL-2 and IFN-γ in splenocytes in the immunized mice. PD was shown to enhance the mRNA expression of IL-2 and IFN-γ, so the effects of PD on the production of Th1 cytokines may result, at least in part, from regulation mRNA expression of these cytokine themselves.

CTLs have been proved to be important in protection from infectious diseases and cancer [30]. Although not essential for protective immunity against HBV, CTL may be important for avoiding or overcoming the chronic carrier state. Indeed, many previously infected individuals, even years after clinical and serologic recovery, have traces of HBV in their blood and have HBV-specific CTL that express activation markers indicative of recent contact with antigen [31]. With spontaneous cell-mediated cytotoxicity, NK cells are also functionally similar to CTLs. NK cells are part of the first line of innate defense against cancer cells and virus-infected cells [32]. In humans, 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 [33]. An important disadvantage of Alum is the induction of a Th2- rather than a Th1-type immune response. In the case of recombinant HBsAg, the use of Alum as an adjuvant appears to interfere with cell-mediated immunity and blocks activation of CTL [10]. In this investigation, addition of Alum to HBsAg did not result in increase in the killing activity of NK cells and HBsAg-specific CTLs in HBsAg-immunized mice. In contrast, PD significantly enhanced the lytic activity of NK cells and specific killing activity of CTLs in mice immunized with HBsAg at three doses ($P < 0.001$), suggesting that the usage of PD in HBsAg vaccine can help to improve specific and non-specific ability to kill HBV.

In conclusion, two important features of PD have been clearly shown: (1) PD is a strong Th1/Th2 adjuvant for the HBsAg in the mice model and (2) the immunogenicity of the PD-adjuvanted hepatitis B vaccine is better than that of a vaccine adjuvanted with Alum. The HBsAg-specific antibody immune response induced by PD vaccine was associated with higher antibody titers than that provided by the Alum vaccine and moreover cell-mediated responses were elicited. These observations suggest that the use of PD as an adjuvant for HBsAg may provide better protection against HBV by eliciting both humoral and cellular immune responses, and that PD should be considered for the development of prophylactic or therapeutic vaccines for which a balanced and potent stimulation of both the cellular and humoral responses to a protein or peptides is required.

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