Platycodin D2 Improves Specific Cellular and Humoral Responses to Hepatitis B Surface Antigen in Mice

by **Yong Xie**[a][b][c], **Shu-Wang He**[a], **Hong-Xiang Sun***[a], and **Duo Li**[b]

[a]) Key Laboratory of Animal Epidemic Etiology & Immunological Prevention of the Ministry of Agriculture, College of Animal Sciences, Zhejiang University, Hangzhou 310029, P. R. China (phone: +86-571-86971091; fax: +86-571-86971091; e-mail: sunhx@zju.edu.cn)

[b]) School of Biosystems Engineering and Food Science, Zhejiang University, Hangzhou 310029, P. R. China

[c]) Department of Pharmacy, Fujian University of Traditional Chinese Medicine, Fuzhou 350108, P. R. China

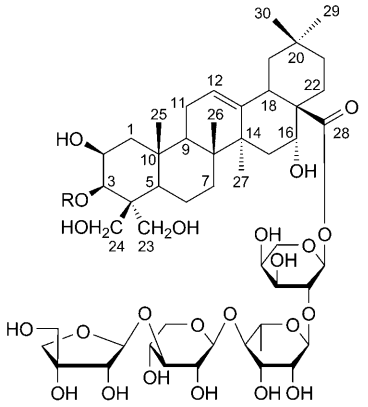
Platycodin D2 (**1**), a less hemolytic saponin from the root of *Platycodon grandiflorum* than platycodin D (**2**), was evaluated for the potential to enhance specific cellular and humoral immune responses to hepatitis B surface antigen (HBsAg) in mice. It significantly increased the concanavalin A (Con A)-, lipopolysaccharide (LPS)-, and HBsAg-induced splenocyte proliferation in HBsAg-immunized mice ($P<0.05$, $P<0.01$, and $P<0.001$, resp.). HBsAg-specific IgG, IgG1, IgG2a, and IgG2b antibody titers in the serum were also markedly enhanced by **1** compared to the HBsAg control group ($P<0.01$ or $P<0.001$). Moreover, **1** significantly promoted the production of Th1 (IL-2 and IFN-γ) and Th2 (IL-4 and IL-10) cytokines from splenocytes in the HBsAg-immunized mice ($P<0.001$). The adjuvant potential of **1** on splenocyte proliferation, serum HBsAg-specific IgG2a and IgG2b antibody response, as well as Th1-cytokine secretion from splenocytes in the HBsAg-immunized mice was higher than that of Alum. The results suggest that **1** could improve both cellular and humoral immune responses to HBsAg in mice. Hence, **1** might be a promising adjuvant for hepatitis B vaccine with dual Th1- and Th2-potentiating activity.

Introduction. – The hepatitis B virus (HBV) is a serious and common virus. No treatment is currently available for acute hepatitis B, and immunization with a vaccine is the most effective approach to prevent HBV infection and its consequences [1]. Hepatitis B surface antigen (HBsAg) adsorbed on aluminum compounds (Alum) is currently the most common vaccine against HBV infection. Though recognized as an effective vaccine, nearly 10% of vaccinees fail to develop protective levels of anti-HBsAg antibodies [2]. Optimization of vaccine effectiveness could be achieved by the use of appropriate adjuvants, which could enhance vaccine coverage of non-responders [3]. 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 [4]. However, Alum, which is the only vaccine adjuvant licensed by the *Food and Drug Administration* (*FDA*) for use in humans and used as adjuvants in the hepatitis B vaccines currently commercialized, has a limited enhancing potential of Th1-type and cell-mediated immune responses [5][6].

Saponins are natural glycosides of steroids or triterpenoids that exhibit many different biological and pharmacological activities [7]. Notably, saponins can activate

the mammalian immune system, which incited interest in their potential as vaccine adjuvants [8]. The most widely used saponin-based adjuvants are Quil A and its derivative QS-21, isolated from the bark of *Quillaja saponaria* MOLINA, which have been evaluated in numerous clinical trials [8]. Their unique potential to stimulate both the Th1-immune response and the production of CTL against exogenous antigens make them ideal for use in subunit vaccines, vaccines directed against intracellular pathogens, and therapeutic cancer vaccines [9][10]. However, Quil A and QS-21 have serious drawbacks such as high toxicity, undesirable hemolytic effects, and instability in aqueous milieu, which limit their use as adjuvant in vaccines [11][12]. In addition, the over exploitation of the bark of *Q. saponaria* has caused important ecological damage and considerable shortage of the available supplies [13]. This has incited research for saponin-based adjuvants from other natural products.

In our previous work, the total saponins from the roots of *Platycodon grandiflorum* were shown to display adjuvant immunological effect [14]. From this extract, four platycodigenin-type saponins with adjuvant activity, *viz*., platycodins D2, D, D3, and platycoside E (**1**–**4**, resp.) were isolated, and their hemolytic activity and adjuvant potentials on the immune responses to ovalbumin (OVA) in mice were investigated [15][16]. Compounds **1**–**4** share a platycodigenin skeleton and the same sugar side chains attached to C(28) of the aglycone. Saponins **2**–**4** only differ from one another by the number of glycosyl units in the sugar moieties attached to C(3). Among these three saponins, the hemolytic activity and adjuvant potential decreased in the order **2** > **3** > **4**. This implicates that the addition of a β-D-glucopyranosyl or β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranosyl group to C(6′) of the glucopyranosyl group at C(3) of the aglycone decreased the hemolytic activity as well as the adjuvant potential [15]. Compound **1** was structurally very similar to **3**, except for the sugar linkage of the disaccharide moiety at C(3) of the aglycone. In **1**, the linkage of the disaccharide is 1 → 3, and in **3**, it is 1 → 6. However, the adjuvant activities of **1** on the cellular and humoral immune responses to OVA in mice were higher than those of **3**, while there was no significant difference between those of **1** and **2**. Thus, the linkage of glycosyl groups in the sugar chain could also have an influence on the activity of platycodigenin-type saponins.



	R
1	Glc-(1→3)-Glc
2	Glc
3	Glc-(1→6)-Glc
4	Glc-(1→6)-Glc-(1→6)-Glc

(Glc = β-D-Glucopyranosyl)

Compound **2** has been shown to be a potent adjuvant of specific immune responses to hepatitis B surface antigen (HBsAg) [17]. However, its hemolytic activity was higher than that of **1**, with concentrations inducing 50% of hemolysis towards 0.5% rabbit red blood cell suspensions being 11.79 ± 0.55 and 18.57 ± 1.37 μg/ml, respectively [15][16]. Aiming at safer and efficient adjuvants for hepatitis B vaccine, the adjuvant potential of **1** on the cellular and humoral immune responses in mice against HBsAg was evaluated here.

Results and Discussion. – 1. *Effect of* **1** *on Splenocyte Proliferation in HBsAg-Immunized Mice.* The cell-mediated immunity plays a key role in virus resolution during HBV infection [18][19]. The capacity to elicit an effective T- and B-lymphocyte immunity can be shown by the stimulation of the lymphocyte proliferation response. It is generally known that concanavalin A (Con A) stimulates T-cell and lipopolysaccharide (LPS) B-cell proliferation. The effects of **1** on mitogen- and HBsAg-stimulated splenocyte proliferation in HBsAg-immunized mice are shown in *Fig. 1*. Con A-stimulated splenocyte proliferation in HBsAg-immunized mice was significantly enhanced by 50–100 μg of **1** ($P < 0.05$ or $P < 0.001$). Compound **1** also markedly promoted the LPS- and HBsAg-induced splenocyte proliferation in mice immunized with HBsAg ($P < 0.05$, $P < 0.01$, or $P < 0.001$). Moreover, mitogen- and HBsAg-stimulated splenocyte proliferation in the mice immunized with **1** at three doses was significantly higher than that in the HBsAg/Alum group ($P < 0.05$, $P < 0.01$, or $P < 0.001$). However, no significant differences ($P > 0.05$) were observed between the HBsAg alone group and the HBsAg/Alum group. These data indicate that **1** was more

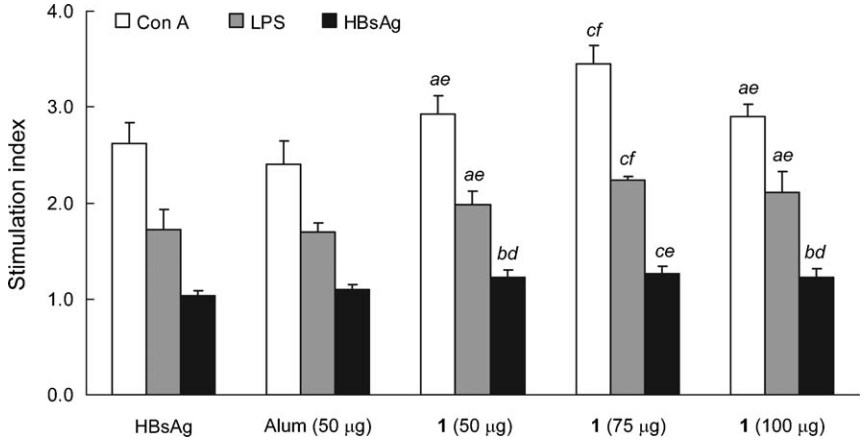


Fig. 1. *Effect of* **1** *on mitogen- and HBsAg-stimulated splenocyte proliferation in mice immunized with HBsAg.* Groups of five female mice were immunized s.c. with 2 μg of HBsAg alone or with 2 μg of HBsAg dissolved in saline containing Alum (50 μg) or **1** (50, 75, and 100 μg) on day 1 and 15. Splenocyte proliferation was measured by the MTT method and shown as a stimulation index (see *Exper. Part*). The values are means ± S.D. ($n = 5$). Significant differences compared to the HBsAg group: $P < 0.05$ (*a*), $P < 0.01$ (*b*), and $P < 0.001$ (*c*). Significant differences compared to the HBsAg + Alum group: $P < 0.05$ (*d*), $P < 0.01$ (*e*), $P < 0.001$ (*f*).

effective than Alum in inducing the activation potential of T and B cells in HBsAg-immunized mice.

2. *Effect of* **1** *on the HBsAg-Specific Serum Antibody Response.* It has been demonstrated that HBsAg-specific antibodies play an important role in preventing HBV infection [20][21]. Therefore, we evaluated the adjuvant activity of **1** on the humoral immune responses to HBsAg. As shown in *Fig. 2*, HBsAg alone induced low levels of serum HBsAg-specific IgG, IgG1, IgG2a, and IgG2b antibodies. However, the addition of **1** to HBsAg at three doses increased the IgG antibody response to levels that were comparable to that induced by Alum. The increase of IgG1, IgG2a, and IgG2b antibody isotypes in response to immunization provides a relative measure of the contribution of Th1 and Th2 immune responses [22]. Both Alum and **1** significantly enhanced the serum HBsAg-specific IgG1 titers in HBsAg-immunized mice ($P<0.01$ or $P<0.001$). However, significant enhancements in HBsAg-specific IgG2a and IgG2b antibody titers were only observed in **1**-immunized mice compared to the HBsAg alone group ($P<0.01$ or $P<0.001$). Indeed, IgG2a and IgG2b antibody titers in the mice immunized with HBsAg/**1** were higher than those in the Alum-treated mice, and there were no significant differences in the serum IgG2a and IgG2b titers between the HBsAg alone and the HBsAg/Alum groups ($P>0.05$). These results suggest that **1** 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 [23].

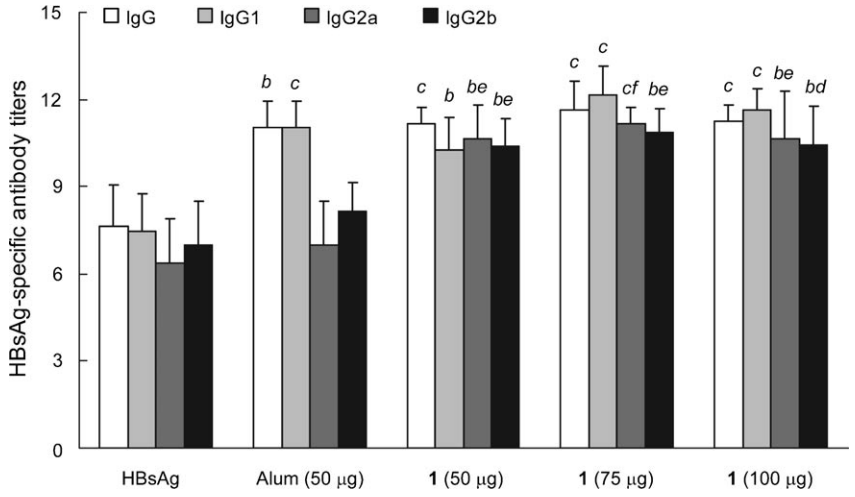


Fig. 2. *Effect of* **1** *on HBsAg-specific IgG, IgG1, IgG2a, and IgG2b antibodies in HBsAg-immunized mice.* Groups of five female mice were immunized s.c. with 2 μg of HBsAg alone or with 2 μg of HBsAg dissolved in saline containing Alum (50 μg) or **1** (50, 75, and 100 μg) on day 1 and 15. HBsAg-specific IgG, IgG1, IgG2a, and IgG2b antibody titers in the sera were measured by an indirect ELISA test (see *Exper. Part*). Results are means ± S.D. ($n=5$). Significant differences compared to the HBsAg group: $P<0.01$ (*b*) and $P<0.001$ (*c*). Significant differences compared to the HBsAg + Alum group: $P<0.05$ (*d*), $P<0.01$ (*e*), $P<0.001$ (*f*).

3. *Effect of* **1** *on Cytokine Secretion by Splenocytes in HBsAg-Immunized Mice.* Adjuvants have significant effects on the nature of the immune responses and can tilt the immune system in favor to Th1- or Th2-type responses [24]. The Th1 immune response in mice is characterized by production of the cytokines IL-2, TNF-β, and IFN-γ, and an enhanced production of IgG2a, IgG2b, and IgG3. The Th2 response is characterized by production of the cytokines IL-4, IL-5, and IL-10, and an enhanced production of IgG1 and secretary IgA. Immunity to different infectious agents requires distinct types of immune responses. The Th1 response is required for protective immunity against intracellular infectious agents, such as viruses, certain bacteria, protozoa, and, presumably, against cancer cells. Th2 Immunity is effective for protection against most bacterial as well as certain viral infections [25][26]. To clearly establish that Th cell-derived cytokines were involved in the adjuvant activity of **1**, we analyzed the Th1/Th2 cytokine secretion profiles in HBsAg-immunized mice using ELISA. As shown in the *Table*, the concentrations of cytokines IL-2, IL-4, IL-10, and IFN-γ in the culture supernatants of HBsAg-stimulated splenocytes from mice immunized with HBsAg/**1** were significantly higher than those in HBsAg control mice ($P<0.001$), suggesting that **1** significantly induced the production of Th1 and Th2 cytokines in the HBsAg-immunized mice. In contrast, Alum only significantly increased the IL-4 and IL-10 production in HBsAg-immunized mice ($P<0.001$). 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 high production of IL-4 and IL-10 corresponded well to the high levels of IgG1 titers. Together, the HBsAg-specific antibody isotypes and cytokine profiles confirm that **1** simultaneously enhanced a Th1/Th2-type immune response, while Alum was associated with predominantly Th2-type immune responses.

Table. *Effect of* **1** *on Cytokine Secretion by Splenocytes in HBsAg-Immunized Mice*

Groups	Cytokines [pg/ml][a])			
	IL-2	INF-γ	IL-4	IL-10
HBsAg	245 ± 11	1676 ± 212	4.89 ± 0.55	738 ± 115
HBsAg + Alum	244 ± 3.5	1771 ± 35	8.64 ± 0.33[b])	1252 ± 92[b])
HBsAg + **1** (75 μg)	383 ± 21[b])[c])	2356 ± 71[b])[c])	12.8 ± 1.55[b])[d])	1352 ± 89[b])

[a]) The concentration of cytokines IL-2, IL-4, IL-10, and IFN-γ in the culture supernatants of HBsAg-stimulated splenocytes were determined by ELISA (see *Exper. Part*). The values are means ± S.D. ($n=5$). [b]) Significant differences compared to the HBsAg group: $P<0.001$. [c]) Significant differences compared to the HBsAg + Alum group: $P<0.001$. [d]) Significant differences compared to the HBsAg + Alum group: $P<0.01$.

In addition, we also compared the adjuvant activities of **1**–**3** on the immune responses to HBsAg in mice. Although the adjuvant activities of **2** on the immune responses to HBsAg were slightly higher than those of **1**, there was no significant difference. However, **3** did not improve specific cellular and humoral response to HBsAg in mice under the same conditions (data not shown). Therefore, **1** has intermediate hemolytic and adjuvant activities on the immune responses to HBsAg.

These results also confirm that the linkage of glycosyl groups in the sugar chain could have an influence on the adjuvant activity of platycodigenin-type saponins.

Conclusions. – Compound **1** significantly enhanced the specific cellular and humoral response to HBsAg and elicited a balanced Th1/Th2-type immune response against HBsAg in mice. Since **1** possessed less hemolytic activity than **2** and an ideal adjuvant property with dual Th1- and Th2-potentiating activity, **1** could be a safe and effective candidate as adjuvant for hepatitis B vaccine.

We gratefully acknowledge the *National Natural Science Foundation of China* (No. 30871888), the *Zhejiang Provincial Natural Science Foundation of China* (No. R3080027), and the *Administration of Traditional Chinese Medicine of the Zhejiang Province* (No. A2006Z017) for financial support.

Experimental Part

General. Purified recombinant hepatitis B surface antigen (HBsAg) from *Saccharomyces cerevisiae* was obtained from *Shenzhen Kangtai Biological Products Co. Ltd*. (Guangzhou, China). Concanavalin A (Con A), lipopolysaccharide (LPS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium hydrobromide (MTT), *RPMI-1640* medium, and rabbit anti-mouse IgG peroxidase conjugate were purchased from *Sigma Chemical Co*. (Saint Louis, Missouri, USA), goat anti-mouse IgG1 and IgG2b peroxidase conjugate from *Southern Biotech. Assoc*. (Birmingham, AL, USA), cytokine (IL-2, IL-4, IL-10, and IFN-γ) detecting ELISA kits from *Wuhan Boster Biological Technology Co. Ltd.* (Hubei, China), fetal calf serum (FCS) from *Hangzhou Sijiqing Corp.*, and aluminum hydroxide gel (Alum) from *Zhejiang Wanma Pharm Co. Ltd.* (Zhejiang, China).

Platycodin D2 (= *Platycodigenin 3*-O-*[β*-D-*Glucopyranosyl-(1→3)-β*-D-*glucopyranosyl]-28*-O-*[β*-D-*apiofuranosyl-(1→3)-β*-D-*xylopyranosyl-(1→4)-α*-L-*rhamnopyranosyl-(1→2)-α*-L-*arabinopyranoside]*; **1**). Platycodin D2 ($C_{63}H_{102}O_{33}$, 1386.63) was isolated from the roots of *P. grandiflorum* according to [16] and its structure was elucidated by spectroscopic analysis including 2D-NMR and HR-ESI-MS spectroscopy [16]. The purity of **1** was determined to be 98.3% by HPLC on a *Waters 600E* HPLC instrument equipped with a *Symmetry C18* column (250 × 4.6 mm i.d., particle size 5 μm), a *Waters 2996 PDA* detector, eluted with MeOH/H_2O 52 : 48 (t_R 21.14 min). The stock soln. of **1** (4 mg/ml) in 0.89% saline was sterilized by filtration (0.22-μm *Millipore* filter) and analyzed for its endotoxin level by a gel-clot *Limulus* amebocyte lysate assay. The endotoxin level in the stock soln. was less than 0.5 endotoxin units (EU)/ml.

Experimental Animals. Female ICR mice (Grade II, 5 weeks old) weighing 18–22 g were purchased from the *Zhejiang Experimental Animal Center* (Hangzhou, China; certificate No. 22-2001001) and acclimatized for 1 week prior to use. Rodent laboratory chow and tap water were supplied *ad libitum*, and the mice were maintained under controlled conditions at 24 ± 1°, 50 ± 10% rel. humidity, and a 12-h light/12-h dark cycle. All the procedures conducted were in strict accordance with the P. R. China legislation on the use and care of laboratory animals and the guidelines established by the Institute for Experimental Animals of the Zhejiang University and were approved by the university committee for animal experiments.

Immunization. Six-week-old female ICR mice were divided into six groups, consisting of five mice each. Animals were immunized subcutaneously (s.c.) on day one with 2 μg of HBsAg alone or with 2 μg of HBsAg dissolved in 0.2 ml saline containing Alum (50 μg) or **1** (50, 75, or 100 μg). Saline-treated animals were included as control. A boosting injection was given two weeks later. Sera and splenocytes were collected two weeks after the second immunization for measurement of specific antibody and cellular immune responses to HBsAg.

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. The erythrocytes were lysed with NH_4Cl (0.8%, *w*/*v*). After centrifugation (380*g* at 4° for 10 min), the pelleted cells were washed

three times with PBS and resuspended in complete medium (*RPMI 1640* supplemented with 12 mM *HEPES* buffer (pH 7.1), 0.05 mM 2-sulfanylethanol, penicillin (100 IU/ml), streptomycin (100 μg/ml), and 10% FCS). Cell numbers were counted with a hemocytometer by the trypan blue dye exclusion technique. Cell viability exceeded 95%. Splenocytes were seeded into four wells of a 96-well flatbottom microtiter plate (*Nunc*) at 5×10^6 cells/ml in 100 μl of complete medium, and Con A (final concentration 5 μg/ml), LPS (final concentration 10 μg/ml), HBsAg (final concentration 4 μg/ml), or medium were added to give a final volume of 200 μl. After incubation at 37° in a humidified atmosphere with 5% CO_2 for 44 (Con A and LPS) or 68 h (HBsAg), 50 μl of MTT soln. (2 mg/ml) were added to each well. The plates were incubated for another 4 h, centrifuged at 1400*g* for 5 min, and the untransformed MTT was removed carefully by pipetting. To each well, DMSO (200 μl) was added, and after 15 min shaking to dissolve the colored material, the absorbance was determined with an ELISA reader (*Model 680*, *Bio-RAD Instruments*) at 570 nm with a 630-nm reference. The stimulation index (*SI*) was calculated by dividing the absorbance of the mitogen-cultures by the absorbance of the non-stimulated cultures.

Measurement of HBsAg-Specific Antibody. HBsAg-Specific IgG, IgG1, IgG2a, and IgG2b antibodies in serum were detected by an indirect ELISA as described by *Xie et al.* [17]. Briefly, the 96-well microplates were coated with 100 μl of HBsAg soln. (2 μg/ml in 50 mM carbonate buffer, pH 9.6) for 24 h at 4°. 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° for 2 h. After three washings with PBS, 100 μl of a series of diluted sera samples or 0.5% FCS/PBS as control were added to triplicate wells. The plates were then incubated for 2 h at 37°, followed by washing (3 × PBS). Aliquots of horseradish peroxidase-conjugated antibody against IgG, IgG1, IgG2a, or IgG2b were added to the wells and incubated for 2 h at 37°. After washing, the peroxidase activity was determined as follows. After the addition of 100 μl of substrate soln. (10 mg benzene-1,2-diamine and 37.5 μl of 30% H_2O_2 in 25 ml of 0.1M citrate/phosphate buffer pH 5.0) to each well, the plate was incubated for 10 min at 37°, and then the enzyme reaction was stopped by adding 50 μl/well of an aq. 2N H_2SO_4 soln. The optical density (*OD*) was measured with an ELISA reader at 490 nm with a 595-nm reference. Data were expressed as the mean *OD* value of the samples minus the mean *OD* value of the blank control. Whenever sets of serum samples have been subjected to within- and between-group comparisons, ELISA assays were performed on the same day for all samples.

Cytokine Measurements in the Supernatants of Cultured Splenocytes by ELISA. Splenocytes (5×10^5 cells/well) from the immunized mice, prepared as described above, were incubated with HBsAg (final concentration of 4 μg/ml) in 24-well culture plates at 37° in 5% CO_2. After 48 h, the plates were centrifuged at 1400*g* for 5 min and culture supernatants were collected for the detection of IL-2, IL-4, IL-10, and IFN-γ levels using commercial ELISA kits. Briefly, the culture supernatants or cytokine standards were added to 96-well flatbottom microtiter plates coated with coating antibody, and the plates were preincubated at 37° for 1.5 (IL-2, IL-4, and IL-10) or 2 h (IFN-γ). Plates were washed, a detecting antibody was added to each well, and then the plates were incubated at 37° for 1 h, before the addition of the avidin-biotin-peroxidase complex (ABC). After incubation for another 30 min, plates were washed and developed with tetramethyl benzidine (TMB) at 37° for 15 min. The reaction was stopped by addition of 100 μl of stop solution. The *OD* was measured with an ELISA reader at 450 nm.

Statistical Analysis. The data were expressed as mean ± S.D. and examined for their statistical significance of difference with ANOVA and the post-hoc test, $P < 0.05$ being considered significant.

REFERENCES

[1] C. W. Shepard, E. P. Simard, L. Finelli, A. E. Fiore, B. P. Bell, *Epidemiol. Rev.* **2006**, *28*, 112.
[2] H. Deulofeut, A. Iglesias, H. Mikael, D. H. Bing, Z. Awdeh, J. Yunis, D. Marcus-Bagley, M. S. Kruskall, C. A. Alper, E. J. Yunis, *Mol. Immunol.* **1993**, *30*, 941.
[3] S. F. Dong, J.-M. Chen, W. Zhang, S.-H. Sun, J. Wang, J.-X. Gu, D. Boraschi, D. Qu, *Int. Immunopharmacol.* **2007**, *7*, 725.
[4] Y. Li, E. R. Kandimalla, D. Yu, S. Agrawal, *Int. Immunopharmacol*. **2005**, *5*, 981.
[5] H. HogenEsch, *Vaccine* **2002**, *20* (Suppl. 3), S34.
[6] R. Schirmbeck, K. Melber, A. Kuhrober, Z. A. Janowicz, J. Reimann, *J. Immunol.* **1994**, *152*, 1110.

[7] S. G. Sparg, M. E. Light, J. van Staden, *J. Ethnopharmacol.* **2004**, *94*, 219.
[8] H.-X. Sun, Y. Xie, Y.-P. Ye, *Vaccine* **2009**, *27*, 1787.
[9] H. Takahashi, T. Takeshita, B. Morein, S. Putney, R. N. Germain, J. A. Berzofsky, *Nature* **1990**, *344*, 873.
[10] S. Soltysik, J.-Y. Wu, J. Recchia, D. A. Wheeler, M. J. Newman, R. T. Coughlin, C. R. Kensil, *Vaccine* **1995**, *13*, 1403.
[11] D. C. Waite, E. W. Jacobson, F. A. Ennis, R. Edelman, B. White, R. Kammer, C. Anderson, C. R. Kensil, *Vaccine* **2001**, *19*, 3957.
[12] G. Liu, C. Anderson, H. Scaltreto, J. Barbon, C. R. Kensil, *Vaccine* **2002**, *20*, 2808.
[13] R. San Martín, R. Briones, *Econ. Bot.* **1999**, *53*, 302.
[14] Y. Xie, H.-J. Pan, H.-X. Sun, D. Li, *Vaccine* **2008**, *26*, 3937.
[15] Y. Xie, Y.-P. Ye, H.-X. Sun, D. Li, *Vaccine* **2008**, *26*, 3452.
[16] Y. Xie, W. Deng, H.-X. Sun, D. Li, *Int. Immunopharmacol.* **2008**, *8*, 1143.
[17] Y. Xie, H.-X. Sun, D. Li, *Vaccine* **2009**, *27*, 757.
[18] B. D. Livingston, J. Alexander, C. Crimi, C. Oseroff, E. Celis, K. Daly, L. G. Guidotti, F. V. Chisari, J. Fikes, R. W. Chesnut, A. Sette, *J. Immunol.* **1999**, *162*, 3088.
[19] M. A. Rico, J. A. Quiroga, D. Subira, S. Castanon, J. M. Esteban, M. Pardo, V. Carreno, *Hepatology* **2001**, *33*, 295.
[20] R. Williams, *Hepatology* **2006**, *44*, 521.
[21] S. Assad, A. Francis, *Vaccine* **1999**, *18*, 57.
[22] F. D. Finkelman, J. Holmes, I. M. Katona, J. F. Urban Jr, M. P. Beckmann, L. S. Park, K. A. Schooley, R. L. Coffman, T. R. Mosmann, W. E. Paul, *Annu. Rev. Immunol.* **1990**, *8*, 303.
[23] T. Germann, M. Bongartz, H. Dlugonska, H. Hess, E. Schmitt, L. Kolbe, E. Kölsch, F. J. Podlaski, M. K. Gately, E. Rüde, *Eur. J. Immunol.* **1995**, *25*, 823.
[24] F. M. Audibert, L. D. Lise, *Immunol. Today* **1993**, *14*, 281.
[25] S. L. Constant, K. Bottomly, *Annu. Rev. Immunol.* **1997**, *15*, 297.
[26] A. S. McKee, M. W. Munks, P. Marrack, *Immunity* **2007**, *27*, 687.

Received January 5, 2009