Vaccine Adjuvant Ginsenosides Rg1 Enhances Immune Responses against Hepatitis B Surface Antigen in Mice

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Vaccine Adjuvant Ginsenosides Rg1 Enhances Immune Responses against Hepatitis B Surface Antigen in Mice

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Abstract

The adjuvant effect of ginsenoside Rg1 on immune responses against Hepatitis B surface antigen (HBsAg) in mice was investigated. Female BALB/c mice were subcutaneously (s.c.) injected with saline or HBsAg antigen with or without Rg1 on 7 days and 21 days. Samples were collected 2 weeks after the boosting for the detection of anti-HBsAg immunoglobulin G (IgG) isotypes in sera and gamma interferon (IFN-γ) and interleukin-4 (IL-4) produced in splenocytes. The innate and adaptive immune responses were measured in mice immunized as described above. The results showed that ginsenosides Rg1 had adjuvant properties in stimulating IgG, splenocyte proliferation, and mRNA expression of cytokines IFN-γ and IL-4 as well as the expression of cell surface marker TLR4 in the HBsAg-immunized mice. These results indicate that Rg1 enhances both Th1 (IgG2b, IFN-γ) and Th2 (IgG1 and IL-4) responses. In addition, TLR4 signaling pathway is involved in the adjuvant activities.
of ginsenosides Rg1.

**Keywords** Ginsenosides Rg1, Hepatitis B surface antigen (HBsAg), immune adjuvant, humoral immunity, cell immunity

**Introduction**

Vaccination is the most effective and valuable tool in the prevention of pathogenic organisms and tumors. Adjuvant is an important component of an effective vaccine. Aluminum adjuvant is the most widely and representatively commercial vaccine adjuvant approved clinically. Unfortunately, increasing studies in human and animals showed that aluminum is a relatively poor adjuvant for antibody induction to recombinant protein antigens, such as HBsAg vaccine. Furthermore, aluminum effectively enhances a Th2-type humoral immune response, and is not highly effective at stimulating Th1-type cell-mediated immune responses which contributes to antiviral and anti-tumor immunity (Jeong et al. 2012; Mahboubi et al. 2012). So it has been developed more potent and reliable immune adjuvants for vaccines over the past decades. Novel adjuvants such as AS04 (Levie et al. 2002) and CpG oligonucleotides (Cooper et al. 2004) have been studied, but have a generally unacceptable and adverse effects including the expense of increased reactionogenicity (Levie et al. 2002) or uncertain safety (Cooper et al. 2004). Hence, developing suitable adjuvants become one of the most significant challenges in vaccination.

In recent years, a large number of clinical and experimental studies had shown that natural products have been a rich and reliable source of compounds for immune adjuvants such as saponins. Previous studies on the immune adjuvant activity of
saponins primarily focused on QuilA extracted from the bark of the tree *Quillaja saponaria* Molina. QS-21 is a representative of QuilA. QS-21 and QuilA (saponin-type adjuvants) induce strong humoral and cellular immune responses. But due to the unstable and easily hydrolyzed nature of QuilA with severe hemolytic and toxic side effects, QuilA thereby limited its use in human vaccines.

Hence, these reason have led to research for alternative adjuvants. Increasing studies have revealed that ginsenoside Rg1 extracted from the root of *Panax ginseng* C. A. Meyer was the most active saponin to have a adjuvant effect (Qu et al. 2011; Su et al. 2015). Although many studies have been revealed about the adjuvant effect of ginsenoside Rg1, the potential mechanisms remain unclear. Toll-like receptors (TLRs) are pattern recognition receptors and play a critical role in the innate and adaptive inflammatory responses to host defense against microbial infection. Recent studies indicate that vaccine adjuvants activate the antigen-presenting cells (APCs) via TLRs signaling pathway (Shima et al. 2013). TLR4 and TLR9 were highly expressed in different immune cells, such as B cells, dendritic cells, macrophages, and specific types of T cells. TLR4 localized in the cell membrane recognizes bacterial lipopolysaccharide (LPS) and plant-derived molecules such as taxol (Yan et al. 2015). The CpG oligonucleotide is recognized by TLR9 in endo/lysosome compartments and triggers the production of Th1-promoting cytokines (Shima et al. 2013).

In the present study, HBsAg was used as a recombinant antigen and we
investigated the effect of ginsenoside Rg1 as an adjuvant of HBsAg vaccine. Hepatitis
B is still one of the vaccine- preventable diseases that threaten human health. Nevertheless, the number of persons who develop protective antibody (anti-HBsAg) against HBV surface antigen (HBsAg) is lower, and the antibody titres of those who mount an antibody response are reduced and declined logarithmically with time. So we explored whether TLRs activation involved in immune responses to the adjuvant of HBsAg vaccine.

Materials and methods

Chemicals and Reagents

Ginseng saponin Rg1 (purity > 98%) was purchased from Chengdu Purechem-Standard Co., Ltd. (Sichuan, China) (Fig. 1). Hepatitis B surface antigen (HBsAg) was purchased from Center For Disease Control of Hubei province (Hubei, China). RPMI 1640 and fetal bovine serum (FBS) were ordered from Gibco (Grand Island, NY, USA). IgG, IgG1 and IgG2b antibody was purchased from eBioscience (USA). Tris-base, Tween-20, potassium carbonate, trypan blue, ConA were purchased from Wuhan kori biotechnology (Wuhan City, Hubei, China). CCK-8 assay kit was ordered from Shanghai East-Chemical Technology Co., Ltd (Jiangsu, China). CD284 (TLR4) (PE labeled) and CD289 (TLR9) (FITC labeled) were purchased from eBioscience (USA). TMB color reagent (A, B color liquid) was purchased from Yichang Baiao Biotechnology (Yichang City, Hubei, China). cDNA reverse transcription and PCR amplification kit were purchased from Takara Co., Ltd.
The polymerase chain reaction (PCR) primers of GAPDH, IFN-γ and IL-4 were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China) (Table 1).

**Animals**

BALB/C mice (female, 6 weeks of age) weighing 18 to 22 g were purchased from the Laboratory Animal Center of Hubei province (Hubei, China) and were kept in polypropylene cages with sawdust bedding in specific pathogen free (SPF) level conditions. The mice were exposed to a 12 h/12 h light/dark cycle at 22 ± 2°C with 60 ± 5 % of relative humidity. Food and water were supplied ad libitum. All animal procedures used for the animals and their care followed the internationally accepted principles as found in the Guidelines for Keeping Experimental Animals issued by the government of China. The researchers received ethical training from Three Gorges University.

**Experimental groups and immunization**

36 Female BALB/C mice were randomly divided into 4 groups: normal control, HBsAg control, HBsAg + Rg1-low, HBsAg + Rg1-high. Each mouse were subcutaneously injected twice at a 2-weeks interval with saline (200 µL) (normal control) or HBsAg (5 µg) in saline solution with or without (HBsAg control) Rg1 (50 µg or 100 µg). Two weeks after the boosting injection, blood samples were collected from the orbital venous sinus for measurement of serum HBsAg-specific IgG. The serum of the blood samples was separated by centrifuging at 3000g for 15 min and stored at −20°C until use. Splenocytes were isolated for determination of lymphocyte
proliferation, cytokine expression.

**CCK-8 assay for lymphocyte proliferation**

Splenocytes prepared from the mice were transferred to RPMI 1640 medium containing 100 IU/mL penicillin, 100 µg/mL streptomycin and 10% fetal bovine serum. Cell viability was estimated using the trypan blue exclusion and the concentration of viable lymphocytes was more than 95%. 500 µL splenocytes were seeded into each well in a 24-well flat-bottom microtiter plate at a concentration of $5.0 \times 10^6$ cells / ml, thereafter complete medium (Negative control), HBsAg (final concentration 5 µg·mL$^{-1}$) or ConA (final concentration 5 µg·mL$^{-1}$) were added giving a final volume of 1 mL to stimulate lymphocyte proliferation. After incubated at 37 °C in a humid atmosphere with 5% CO$_2$ for 72h, 100 µL of CCK-8 solution was added and incubated for 2 h, and read immediately at OD450 nm by microplate reader, calculating the stimulation index (SI) based on the formula: $SI = (OD_{experimental\; group} - OD_{blank}) / (OD_{negative\; control} - OD_{blank})$.

**Determination of serum IgG, IgG1 and IgG2b by ELISA**

An indirect enzyme-linked immunosorbent assay (ELISA) was conducted to measure the titers of anti-HBsAg antibodies in serum as previously described by Su et al. (Su et al. 2012). Briefly, flat-bottomed 96-well microtiter plates were coated with 100 µL/well of diluted HBsAg (5 µg/mL) in PBST and were incubated at 4°C for 16 h. After washing with PBST for 5 min, the plates were blocked with 200 µL/well 1% BSA diluted in PBST for 1h at 37 °C. Then, blocking buffer being removed, the wells
were washed three times (5 min/each) with PBST. To measure IgG, IgG1 and IgG2b, 100 µL of diluted serum samples (1:9000) with 1% BSA was applied to the plates and the plates were incubate for 1 h at 37°C. After another washing, 100 µL of biotinylated goat anti-mouse secondary antibody (IgG, IgG1, or IgG2b) diluted in 1% BSA (1:3000) was added and incubated for 50 min at 37°C. Plates were washed again, and 100 µL of TMB color reagent was added to each well and further incubated for 15 min in dark. The reaction was stopped finally using 50 µL of 2M H$_2$SO$_4$. The optical density (OD) of the plates was read immediately at 450 nm.

**Quantification of IFN-γ and IL-4 genes by reverse transcription PCR (RT-PCR)**

Splenocytes were prepared and treated as same as above, then cells were centrifuged 10 min (380g at 4°C), and washed in ice-cold PBS, then subjected to RNA extraction. Splenocytes were lysed in 1 mL of Trizol reagent (Takara, Japan) and total RNA was extracted from splenocytes according to the manufacture’s protocol. The cDNA was synthesized from total RNA. Reverse-transcription reaction was performed by mixing 1 µg of RNA with 5 µL PrimeScript reagent (Takara) in a DEPC-treated tube, thereafter the final volume was adjusted to 20 µL with RNase Free dH$_2$O. The reverse-transcription reaction was performed in a condition of 15 min at 37°C, 5 sec at 85°C, stored at 4°C. The polymerase chain reaction was performed using 1 µL cDNA up to a final volume of 25 µL reaction in a Bio-Rad iQ5 96-well plate. 1 µL of GAPDH, IFN-γ and IL-4 primer were amplified at a concentration of 12.5 µL TaKaRa 2×PCR Master Mix and 10.5 µL RNase Free dH$_2$O. An initial activation at 94°C for 5 min was followed by an amplification target sequence of 29 cycles of 94°C for 30 s,
55°C for 30 s, 72°C for 30s and 72°C for 5 min in a thermocycler (Bio-RadMJ MiniPCR, USA).

**FCM assay for the expression of TLR4 and TLR9**

The cell surface receptor staining was evaluated using flow cytometry (Sobol et al. 2011). Briefly, Splenocyte suspensions were collected as above, thereafter the splenocytes were washed with PBS and stained for cell surface markers with respective antibodies (TLR4, TLR9) for 30 min at 4 °C in dark according to the instructions of manufacturer. 500 µL cells in PBS were analysed by flow cytometry (Becton,Dickinson and Company,USA).

**Statistical analysis**

Results were expressed as means ± standard deviation (SD). All analysis was performed with GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA). Multigroup comparisons were analyzed by one-way analysis of variance (ANOVA) test with post hoc contrasts test. $P$ value less than 0.05 were considered as statistically significant.

**Results and discussion**

**Ginsenoside Rg1 enhance the HBsAg antibody production of specific IgG , IgG1 and IgG2b**

The effects of ginsenoside Rg1 on HBsAg -specific IgG, IgG1 and IgG2b antibody production were examined using the methods as above. Different diluted serum
samples were detected for the HBsAg-specific IgG levels. As shown in Fig. 2A, the OD value of serum samples were decreased with the increase of the dilution ratio of serum samples. The positive correlation was demonstrated between the serum dilution and its OD value. The results of Fig. 2B showed that the mean value of the HBsAg antibody titre from the animals immunised with the vaccine. The mean value of IgG, IgG1 and IgG2b in HBsAg alone group were significantly higher than those of the mice injected with the saline. The total levels of IgG antibody were elevated significantly in the HBsAg/50 µg Rg1 group compared with the HBsAg alone group. The IgG1 subtype is considered to be associated with Th2-dominated immune responses, whereas IgG2b is reported to be a mediator of Th1-type immunity (Kawase et al. 2011). The results in the Fig. 2B showed that the treatment with HBsAg/50 µg Rg1 markedly increased the production of the HBsAg-specific IgG1 and IgG2b isotypes compared with the HBsAg group, and significantly favoured the production of IgG2 over IgG1 antibodies. It is because that the adjuvant switch isotype of the antibodies via the appropriate cytokine milieu and that response may transform according to the antigen and the species (Avramidis et al. 2002). These results indicate that the Th1 and Th2 immune responses were induced by the HBsAg/ Rg1 vaccine in vivo and that the ginsenosides Rg1 used as an adjuvant of HBsAg vaccine exerted an adjuvant effect on enhancing the secretion of its specific antibodies and improving the immunogenicity of HBsAg by Th1-mediated cellular immunity and Th2-mediated humoral immunity.

**Ginsenosides Rg1 significantly enhanced lymphocyte proliferation and IFN-γ**
and IL-4 expression

To measure the effect of ginsenosides Rg1 on HBsAg-immunized mice, the lymphocyte proliferation analyzed in the HBsAg-immunized mice. The spleen is vital part of the lymphatic system. Immune function (through phagocytosis, but also through T cell-mediated immunity and B cell-mediated humoral immunity) is the most important function of the spleen (Tarantino et al. 2011). The splenocytes from the immunized mice were prepared to assess the proliferative immune responses to ConA or HBsAg (Fig. 3A). It was observed that the splenocytes from the mice immunized with HBsAg/Rg1 showed a significant proliferative response to ConA or HBsAg. After ConA or HBsAg stimulation lymphocyte proliferation in the mice immunized with HBsAg/Rg1 was significantly higher than in the HBsAg groups ($P < 0.05$). In addition, we determined that the mRNA expression of IFN-γ, IL-4 cytokines of the splenocytes by reverse transcription PCR. Naive helper T cells differentiate into Th1 and Th2, when stimulated with cognate antigens by APCs. Th1 cells secrete IFN-γ and primarily promote cellular immunity. Th2 cells mainly produce IL-4 and promote humoral immunity. As can be seen in Fig. 3B and C, no significant differences in the mRNA expression of cytokines IFN-γ and IL-4 between the mice immunized with saline and HBsAg alone. But the mRNA expression of IFN-γ and IL-4 cytokines were up-regulated in HBsAg/Rg1 stimulation compared with HBsAg alone group, indicating that ginsenosides Rg1 simultaneously induced the gene expression of the Th1 and Th2 cytokines in splenocytes upon stimulation of HBsAg, and had immuno-adjuvant activities capable of boosting both cellular (Th1) and
humoral (Th2) immune responses.

**Influences of surface receptor TLR4 and TLR9**

In order to detect the mechanism about the adjuvant effect of ginsenoside Rg1, we hypothesized that the adjuvant effects of Rg1 may be related to TLR signaling pathways. It had been found that many vaccine adjuvants through the activation of TLRs can induce the body to produce interferon and other inflammatory cytokines (Oh et al. 2014; Orr et al. 2014). Therefore we evaluated that the expression of TLR4 and TLR9 to determine if ginsenosides Rg1 had an adjuvant effect on HBsAg by activating TLR4 and TLR9 receptor. In Fig. 4, HBsAg enhanced the expression of cell surface receptor TLR4 and TLR9 in the mice immunized with HBsAg alone compared with the control group. Besides, HBsAg plus ginsenosides Rg1 had significant effect on the expression of TLR4 compared with the HBsAg alone, suggesting that ginsenosides Rg1 had an immuno-adjuvant activities by activating TLR4 receptor. Several studies have reported that the combination of multiple TLR ligands synergistically stimulated APCs and exerted adjuvant effects. Biologically derived oligodeoxynucleotides (ODNs) containing unmethylated CpG sequences (CpG ODNs) are known as an immune adjuvant that are recognized by TLR9 in endo/lysosome compartments. The recognition of CpG ODN by TLR9 triggers the secretion of Th1-promoting cytokines, and finally induces Th1-biased cellular innate and adaptive immunity. Cooper CL and co-workers (Cooper et al. 2004) observed that addition of CpG oligonucleotides (ODN) to stimulate TLR9 signaling increased
hepatitis B virus-specific Ab titers in Engerix-B vaccinated humans. In addition to TLR9 ligands, several studies describe the evaluation of TLR4 ligands used either alone or in combination with other adjuvant formulations. Fei Su et al. (Su et al. 2012) reported that the adjuvant activities of ginsenosides Rg1 and Re extracted from the root of *Panax ginseng C.A. Meyer* were TLR4-dependent in the OVA-immunized mice. Pouliot K, et al. (Pouliot et al. 2014) also suggested TLR4 and MyD88 were necessary for a strong humoral and cell-mediated immune response in mice immunized with DP6-001 vaccine adjuvanted with MPLA. These results showed that TLR4 activation in the vaccine adjuvant was same as our results.

**Conclusion**

In summary, our data illustrate that ginsenosides Rg1 exhibits a range of immunological adjuvant effects on a number of cell types in the HBsAg-immunized mice. It has been seen that the adjuvant activity of ginsenosides Rg1 produces a high degree of IgG2 antibody and elicits Th1 and Th2 immune response via TLR4 signal pathway. Therefore, these results provide promising road in the future for the development of a novel immune adjuvant aagainst HBV. Ginsenosides Rg1 will be used in future HBsAg vaccination trials to enhance the immune response in human.

**Declarations**

**Conflicts of Interest**

The authors report no conflicts of interest

**Fundings**

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PMID:21119618.


PMID:22472794.


Fig. 1  Chemical structure of ginsenoside Rg1 (C42H72O14; molecular weight, 801.02)

Fig. 2  Antibody titer dilution standard curve (A) and effect of ginsenosides Rg1 on HBsAg-specific IgG, IgG1 and IgG2b antibody levels in serum (1:9000) in HBsAg-immunized mice (B). Mice (n = 9/group) were s.c. immunized with saline or HBsAg antigen with or without Rg1 on 7 days and 21 days. Sera were collected and HBsAg-specific IgG, IgG1 and IgG2b antibody levels in serum were measured by an indirect ELISA. The values were presented as mean ± SD. **P <0.01 vs normal group, #P <0.05, ##P <0.01 vs HBsAg group.

Fig. 3  Effect of ginsenosides Rg1 on the lymphocytes proliferation treatment with ConA and HBsAg stimulation by CCK-8 assay (A) and effect of ginsenosides Rg1 on the mRNA expression of cytokines IFN-γ and IL-4 by RT-PCR (B and C) in splenocytes of HBsAg-immunized mice in vitro. Mice (n = 9/group) were subcutaneously injected with saline or HBsAg with or without Rg1 on 7 days and 21 days. Splenocytes were prepared 2 weeks after the second immunization and cultured with RPMI 1640 medium. Data were expressed as mean ± SD. *P <0.05 vs normal group, #P <0.05, ##P <0.01 vs HBsAg group.

Fig. 4  Effect of ginsenosides Rg1 on the expression of TLR4 and TLR9 receptor in splenocytes. Mice (n = 9/group) were s.c. immunized with saline or HBsAg with or without Rg1 on 7 days and 21 days. Splenocytes were collected after boosting and stained with FITC-conjugated anti-TLR4/anti-TLR9 antibodies for 30 min in the dark. TLR4 and TLR9 receptor levels were examined by flow cytometry. The numbers indicate the percentage of cells in the quadrant. The results were presented as mean ± SD(n = 9). **P <0.01, *P <0.05 vs normal group, #P <0.05 vs HBsAg group.
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Fig 4 Effect of ginsenosides Rg1 on the expression of TLR4 and TLR9 receptor in splenocytes. Mice ($n = 9$/group) were s.c. immunized with saline or HBsAg with or without Rg1 on 7 days and 21 days. Splenocytes were collected after boosting and stained with FITC-conjugated anti-TLR4/anti-TLR9 antibodies for 30 min in the dark. TLR4 and TLR9 receptor levels were examined by flow cytometry. The numbers indicate the percentage of cells in the quadrant. The results were presented as mean ± SD ($n = 9$). **$P < 0.01$, $P < 0.05$ vs normal group, *$P < 0.05$ vs HBsAg group.
Table 1 PCR Primers

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