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Enhancement of humoral and cellular responses to HBsAg DNA vaccination by immunization with praziquantel through inhibition TGF-β/Smad2,3 signaling

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ABSTRACT

Praziquantel (PZQ), which is used to treat all forms of schistosomiasis, has been shown to induce strong T cell activities and decrease T regulatory cell levels. In our study, we investigated whether PZQ may be used as an adjuvant for a hepatitis B surface antigen (HBsAg) DNA vaccine (pcD-S2) in eliciting strong humoral and cellular responses. Our data demonstrate that PZQ as an adjuvant increased T cell proliferation and an HBsAg-specific antibody response that was characterized by a higher ratio of IgG2a/IgG1. Moreover, a higher level of IFN-γ in $CD4^+$ and $CD8^+$ T cells were elicited. In addition, a significantly antigen-specific cytotoxic T lymphocyte response was also observed. The expression of TGF-β can be induced by HBsAg, while PZQ as an adjuvant can inhibit the expression of TGF-β and TGF-β/Smad2,3 signaling. The frequency of $CD4^+CD25^+Foxp3^+$ Treg cells was reduced. Importantly, the regulatory function of $CD4^+CD25^+$ Treg cells was correspondingly impaired. Together, these results suggest that PZQ can enhance humoral and cellular responses to HBsAg DNA vaccination through inhibition TGF-β/Smad2,3 signaling.

1. Introduction

Hepatitis B is a liver disease that results from infection with the hepatitis B virus, which is a worldwide health problem [1,2]. More than 350 million people worldwide may have chronic hepatitis B and about 1–2 million die annually [3,4]. Although commercial recombination HBsAg vaccines and therapeutic drugs are used to prevent and treat chronic HBV infection, they are unable to help clear infected cells [5].

DNA vaccination, a novel vaccine technology that has great potential for reducing infectious disease, induces a strong humoral and cellular immune response [6]. To date, HBV DNA vaccine has been shown to induce strong humoral and cell-mediated immunity in animal models [7,8]. HBsAg DNA vaccine has been shown to clear circulating HBsAg and decrease the amount of HBV mRNA in the liver of transgenic mice [9]. However, plasmid DNA immunogens can elicit cellular immune responses in small laboratory animals, the limited potency in human clinical trials is the concern [10,11]. Accordingly, an effective strategy has been proposed to enhance the immunogenicity of plasmid DNA vaccines through using adjuvant [12–14].

Praziquantel (PZQ) has an excellent record of efficacy and safety for the treatment of infections caused by all forms of schistosomiasis [15]. The humoral immune responses of host can be enhanced after exposure to the drug [16]. In addition, schistosome antigen-specific changes in IL-4, IL-5, IL-10, and IL-13 responses have been observed on treatment with PZQ [17]. Furthermore, evidence shows that PZQ can affect Treg cells and the level of Treg cells decreases in people infected with *Schistosoma mansoni* on effective treatment by the drug [18]. However, whether the PZQ has adjuvant effects on host immune responses remains unclear.

In our study, we demonstrate that the adjuvant effect of PZQ as a potent chemical adjuvant for HBV DNA vaccines in immunized mice, which affects both humoral and cellular responses. Interestingly, negative effect of PZQ on the expression of TGF-β and down-regulation of TGF-β/Smad2,3 signaling suggests that one of its adjuvant effects may be via suppression of immunoregulation, such as to down-regulate the Treg function.

2. Materials and methods

2.1. Reagents and animals

Praziquantel (NCPC, Hebei, China) was initially dissolved in ethanol vehicle and subsequently diluted to 1.0%, 0.5% and 0.25% with the saline solution. The CHO cells expressing recombinant HBsAg (rHBsAg) was kindly provided by China North Pharmaceutical Group Corporation (NCPC, Hebei, China). The HBsAg-derived peptide S208–215 (ILSPFLPL; H-$2K^b$-restricted) were synthesized

Abbreviations: RT-PCR, reverse transcriptase PCR; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; BSA, bovine serum albumin; CFSE, carboxyfluorescein succinimidyl ester.

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by GL Biochem Co., Ltd. (Shanghai, China). Adult female C57BL/6 mice at 8–10 weeks of age were purchased from Animal Institute of Chinese Medical Academy (Beijing, China) and feed with pathogen-free food and water in a 12 h light-cycle condition.

2.2. Plasmid construction and preparations

The pcD-S2 encoding HBV surface antigen preS2 and S was constructed into pcDNA3.0 as described previously [12]. The plasmids were maxi-prepared by the alkaline method as described previously [19], subsequently purified by PEG8000 precipitation, and diluted in saline solution.

2.3. Immunization

The C57BL/6 mice were randomly divided into nine groups ($n = 6$ each), and immunized intramuscularly on days 0, 14, and 28 with either pcD-S2 alone, pcD-S2 combined with different concentrations of PZQ, or with vehicle listed in Table 1. Serum samples were bled both before and after immunization at 2-week intervals.

2.4. Antibody ELISA

Serum samples were analyzed by ELISA on day 7 after the third immunization. The international units of the total IgG were measured with an enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions (SIIC Kinghaw Biotech Co., Ltd., Beijing, China) and the amount of total anti-HBsAg antibody was calculated as previously described [12]. Anti-HBsAg-specific IgG2a and IgG1 were quantified by ELISAs as previously described [12]; the concentrations for IgG1 and IgG2a isotypes were determined according to optical density (OD) values of the diluted samples on the standard curve, which was plotted by the matched mouse IgG isotype with serial dilutions (in steps of 2-fold) from 20 to 0.01 ng/ml, and multiplying by the dilution factor (100-fold). Peroxidase-labeled rat anti-mouse IgG1 and IgG2a (Southern Biotechnology Associates, USA) were used for the enzymatic reaction and detections.

2.5. T cell proliferation

Single lymphocyte suspensions were obtained from spleens of the mice on day 7 after the third immunization. Cells in RPMI-1640 medium (Gibco, Eggenstein, Germany)/10% FBS were used to perform the T cell proliferation by MTT method after the HBsAg stimulation in vitro for 48 h. This method was performed according to the previously described protocols [12]. The OD values were read at 490 nm by a plate reader (Magellan, Tecan Austria GmbH). Data were expressed as stimulation index (SI), calculated as the mean reading of triplicate wells stimulated with an antigen, divided by the mean reading of triplicate wells stimulated with the medium.

Table 1
Immunization groups.

Groups	DNA vaccine	Adjuvant
1	Naïve	
2	100 μg pcDNA3	
3		0.5% PZQ
4		15% ethanol
5	100 μg pcD-S2	
6	100 μg pcD-S2	15% ethanol
7	100 μg pcD-S2	0.25% PZQ
8	100 μg pcD-S2	0.5% PZQ
9	100 μg pcD-S2	1.0% PZQ

Note: PZQ is easily soluble in ethanol and 0.2 mg PZQ was dissolved into 3 ml ethanol. The saline solution of 0.25%, 0.5% and 1.0% PZQ contained 15% ethanol. 15% ethanol and pcD-S2 were control.

2.6. In vivo CTL assay

In vivo cytotoxic assay was performed as described previously [12] with the use of splenocytes from naïve C57BL/6 mice pulsed with 10^{-6} M HBsAg CTL peptide S208–215 [12] and labeled with a high concentration of CFSE (15 μM, $CFSE^{high}$ cells) as target cells. A portion of the same splenocytes was labeled with a low concentration of CFSE (0.5 μM, $CFSE^{low}$ cells) without peptide pulse as a non-target control. The target and control cells were mixed in a 1:1 ratio and injected into immunized mice at 2×10^7 total cells per mouse via the tail vein on day 7 after the third immunization. Four hours later lymph nodes and the spleens of injected mice were removed and the target and control cells were analyzed by their differential CFSE fluorescent intensities using a FACSCalibur (BD Biosciences, USA). Specific lysis was calculated using the following formula: ratio = percentage of $CFSE^{low}$/percentage of $CFSE^{high}$. Percentage of specific lysis = 1 − (ratio unprimed/ratio primed) × 100.

2.7. Flow cytometric analysis

T cells were isolated on day 7 after the third immunization. T cells at 0.5×10^6 cells/20 μl were stimulated in 96-well plates with HBsAg (5 μg/ml) or S208–215 (ILSPFLPL; H-$2K^b$-restricted at 10 μg/ml) and anti-CD28 (5 μg/ml) mAb for 6 h at 37 °C and 5% CO_2. Monensin (2 μg/ml) was added for the last 4 h and the cells were washed three times with PBS/10% FCS. Cells were blocked with Fc-Block (BD Phamingen, San Diego, USA) in PBS for 30 min at 4 °C before fixed with 4% paraformaldehyde and permeabilized with saponin, immunostained with isotype controls, or double stained with anti-CD4-FITC and anti-IL-4-PE, or anti-CD8-PE and anti-IFN-γ-FITC, or anti-CD4-PE and anti-IFN-γ-FITC, or anti-CD4-FITC, anti-CD25-PE and anti-Foxp3-APC for 30 min at 4 °C. The cells were washed and analyzed with a FACSCalibur using the Cell Quest Pro Software (BD Bioscience).

2.8. Mixed lymphocyte reaction (MLR)

Three days following the first immunization, $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells from splenocytes of C57/B6 mice were isolated by MACS sorting (R&D Systems, Inc., Huntingdon Valley, PA, USA). The regulatory function of these $CD4^+CD25^+$ T cells was determined by co-culturing in a MLR. The $CD4^+CD25^+$ T cells were mixed with $CD4^+CD25^-$ T effector cells at various ratios, 1:1, 1:5, and 1:10, stimulated with antigen-presenting cells (APC) from BALB/c mice at the ratio of APC to $CD4^+CD25^-$ T cells 1:3 after the APC was treated with 30 μg/ml mitomycin C. T cell proliferation was detected as described by T cell proliferation assay above.

2.9. RT-PCR

Total RNA was extracted from total splenocytes or from sorted T cells and then was reverse-transcribed. The sequences of the primers are listed in Table 2. Samples were run by 1.5% agarose gel and visualized by stained with EtBr.

2.10. Statistical analysis

Results are presented as means ± S.E.M. Student's t test analysis was used for data analysis. A value of $p < 0.05$ was considered to be statistically significant.