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Improved immune response to an attenuated pseudorabies virus vaccine by ginseng stem-leaf saponins (GSLS) in combination with thimerosal (TS)

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ABSTRACT

Vaccination using attenuated vaccines remains an important method to control animal infectious diseases. The present study evaluated ginseng stem-leaf saponins (GSLS) and thimerosal (TS) for their adjuvant effect on an attenuated pseudorabies virus (aPrV) vaccine in mice. Compared to the group immunized with aPrV alone, the co-inoculation of GSLS and/or TS induced a higher antibody response. Particularly, when administered together with GSLS-TS, the aPrV vaccine provoked a higher serum gB-specific antibody, IgG1 and IgG2a levels, lymphocyte proliferative responses, as well as production of cytokines (IFN-γ, IL-12, IL-5 and IL-10) from lymphocytes, and more importantly provided an enhanced cytotoxicity of NK cells and protection against virulent field pseudorabies virus challenge. Additionally, the increased expression of miR-132, miR-146a, miR-147 and miR-155 was found in murine macrophages cultured with GSLS and/or TS. These data suggest that GSLS-TS as adjuvant improve the efficacy of aPrV vaccine in mouse model and have potential for the development of attenuated viral vaccines.

1. Introduction

Pseudorabies (Pr) is a serious zoonotic infectious disease of livestock and wild mammals. It is caused by pseudorabies virus (PrV), and characterized by a high mortality rate in piglets in non-immune sows, ataxia, respiratory distress as well as abortion et al. in older pigs (Dong et al., 2014; Zuckermann, 2000). Vaccination with attenuated pseudorabies virus (aPrV) is one of the most effective approaches to Pr control. However, poor immune response to vaccination often results in incomplete protection. (Li et al., 2008; Yu et al., 2014). During recent years, the disease was reported to outbreak in a large number of vaccinated swine herds (An et al., 2013), emphasizing the limitations of current vaccines and a demand for the improvement of PrV vaccines.

Our previous studies showed that saponins extracted from the root (GS) or stem-leaf (GSLS) of *Panax ginseng* C.A. Meyer had exhibited adjuvant property. Rivera et al. (2003b) observed that addition of GS in the porcine parvovirus (PPV) vaccine caused significantly more haemagglutination inhibition (HI) titers than PPV vaccine used alone in guinea pigs. Hu et al. (2003) found that a commercial *Staphylococus aureus* bacterin combined with GS induced significantly higher antibody responses in cattle. Rivera et al. (2003a) reported an enhanced humoral response in pigs when a bivalent commercial vaccine to PPV and *Erysipelothrix rhusiopathiae* was supplemented with GS. A synergistic adjuvant effect has been found when GSLS was used with oil adjuvant or GS was used with aluminum hydroxide or rapeseed oil (Song et al., 2009; Zhang et al., 2014).

The aPrV vaccine is usually supplied in a form of lyophilized powder and diluted with physiological saline solution before administration. We hypothesized that the vaccine diluted in a GSLS-containing solution may induce higher immune response than that diluted in physiological saline solution. Because the adjuvant activity of GSLS will significantly diminish after heat sterilization (data not shown), a conventional preservative thimerosal (TS) was added in the GSLS-containing solution. The present study was designed to evaluate GSLS in combination with TS for its adjuvant effect on an aPrV vaccine by measuring the humoral and cellular immunity as well as protection against a virulent field PrV challenge in mice.

Abbreviations: APrV, attenuated pseudorabies virus; FPrV, virulent field pseudorabies virus; GS, ginseng root saponins; GSLS, ginseng stem-leaf saponins; TS, thimerosal.

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2. Materials and methods

2.1. Cells and virus

Porcine kidney (PK-15) and Murine lymphoma (YAC-1) cells were cultured in RPMI-1640 (Hyclone, Logan, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone) and penicillin–streptomycin. Murine macrophages (RAW264.7) were cultured in High Glucose DMEM (Hyclone) supplemented with 10% FBS. A virulent field pseudorabies virus strain (fPrV) was kindly supplied by Zhejiang Academy of Agricultural Sciences; virus was propagated and tittered on PK-15 cells.

2.2. Adjuvant and vaccine formulations

Standardized ginseng stem-leaf saponins (GSLS) was purchased from Hongjiu Ginseng Industry Co. Ltd. (Jilin, China) and contained Re (16.36%), Rd (9.0%), Rg1 (6.0%), Rb2 (3.8%), Rc (3.7%), Rb1 (2.4%) and Rf (0.1%) according to HPLC analysis. Thimerosal (TS) was a product of Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). GSLS and/or TS were dissolved in physiological saline solution at various concentrations (GSLS 0, 4, 6 or 8 μg/100 μl alone or in combination with TS 1 μg/100 μl), and sterilized by passage through 0.22 μm filters. The solutions had less than 0.5 EU/ml of endotoxin and were used to dilute attenuated pseudorabies virus (aPrV; Bartha-K61) vaccine supplied by Jianliang Veterinary Biological Preparations Co. Ltd. (Hangzhou, China).

2.3. Immunization and sample collection

Female ICR mice aged 6–8 weeks were obtained from Shanghai Laboratory Animal Center (SLAC) Co. Ltd. (Shanghai, China), and were maintained under a clear air condition. All the animals were treated following the local national Ethical Principle and Guidelines for Animal Experimentation. In experiment A, groups of mice ($n = 6$) were inoculated intramuscularly (i.m.) twice with aPrV (600 $TCID_{50}$) vaccine having GSLS (4, 6, 8 μg), TS (1 μg), or GSLS (4, 6, 8 μg) in combination with TS (1 μg) on days 1 and 14. Non-immune animals served as a control. Sera were taken at 2 and 3 weeks post boost immunization to analyze antibody responses. In experiment B, groups of mice ($n = 18$) were i.m. immunized with aPrV (1800 $TCID_{50}$) vaccine without or with GSLS-TS on days 1 and 14, non-immune animals served as a control. Twenty-four hours post the first immunization, groups of animals ($n = 6$) were euthanized and splenocytes were isolated for determination of cytolytic activity; blood samples were collected for measurement of IFN-γ. Two weeks post boost immunization, groups of animals ($n = 6$) were euthanized and splenocytes were prepared to analyze lymphocyte proliferation; the supernatants were collected to test the cytokine production after the splenocytes were cultured with 5×10^5 $TCID_{50}$ of heat-inactivated fPrV for 48 h. Sera from mice ($n = 6$) were collected at 0, 1, 2, 3 and 4 weeks post boost immunization to analyze IgG and the isotype responses.

2.4. Mouse challenge test

To examine the effect of GSLS-TS on the protection induced by a PrV vaccine, mice ($n = 10$/group) were i.m. injected with aPrV (1800 $TCID_{50}$) vaccine, GSLS-TS or aPrV vaccine + GSLS-TS on days 1 and 14. Injection of saline solution served as a control. The mice were challenged at 2 weeks post boost immunization by intraperitoneal injection of fPrV at a lethal dose of 5×10^5 $TCID_{50}$. Animal behaviors and survivals were observed for 10 days.

2.5. Quantification of microRNAs in macrophages

RAW264.7 cells were placed at 2×10^6 in 2 ml High Glucose DMEM medium containing 10% FCS in 5% CO_2 at 37 °C. After adherence was formed, 20 μl saline solution, GSLS and/or TS solution were added in triplicates to produce final concentrations of GSLS 60 μg/ml and/or TS 10 μg/ml. For microRNAs quantification, miScript PCR system (Qiagen, Hilden, Germany) were applied to analyze microRNAs expression in RAW264.7 cells stimulated by GSLS and/or TS for 2 and 5 h. The total RNA was isolated with miNeasy Mini Kit complying with the protocol, and miScript® II RT Kit (Qiagen) was used to polyadenylate and convert 1 μg of total RNA enriched with microRNAs to cDNA using an oligo (dT) primer containing a universal tag and miScript Reverse Transcription mix. Thereafter amplified product was detected with miScript SYBR® Green PCR kit, using a primer complementary to the universal tag and miScript primers specific for microRNAs (miR-132, 5′-GGTAACAGTCTACAGCCATGG-3′; miR-146a, 5′-GGGTGAGAACTGAATTCCATGG-3′; miR-147, 5′-GTGTGCGGAAATGCTTCTG-3′; miR-155, 5′-GGGGTTAATGCTAATTGTGATAGG-3′; U6 forward were 5′-CTCGCTTCGGCAGCACA-3′ and U6 reverse were 5′-AACGCTTCACGAATTTGCGT-3′). RT-PCR was performed in triplicate for each sample on ABI 7300 (PE Applied Biosystems, USA) in 20 μl with a concomitant reaction condition (15 min at 95 °C, then followed by 40 cycles of 15 s at 94 °C, 30 s at 55 °C and 30 s at 70 °C). Relative expression levels of microRNAs normalized to U6 were analyzed using the $2^{-\Delta\Delta CT}$ method and represented as fold changes between the samples and the controls.

2.6. Cytotoxic activity

Cytotoxic activity was analyzed as previously described (Cao et al., 2008). In brief, YAC-1 cells (100 μl) as target cell were added into 96-well plates at 2×10^5/well. Splenocytes as effector cell were seeded into the plates at 5×10^6 and 1×10^7 cells per well, providing a final volume of 200 μl containing the effector to target (E: T) ratios at 25:1 and 50:1, respectively. The plates were then incubated at 37 °C for 5 h in 5% CO_2 atmosphere. Subsequently, MTT method was used and the percentage of specific lysis was calculated following the formula: (1- (OD_{570} value of effector and target cells− OD_{570} value of effector cells)/OD_{570} value of target cells) × 100%.

2.7. Serum antibodies

The serum PrV gB-specific IgG was measured using a blocking ELISA kit (IDEXX, Westbrook, USA) according to the manufacturer's instructions. Antibody blocking rate was computed based on the formula: (1-S/N) × 100%; S = OD_{650} value of sample, N = OD_{650} value of negative control. The serum PrV gB-specific IgG1 and IgG2a were analyzed using the same procedure with HRP-conjugated goat anti-mouse IgG1 or IgG2a (1:1000) (Santa Cruz Biotechnology, Inc., USA) used to replace the enzyme-conjugated anti-gB monoclonal antibody.

2.8. Lymphocyte proliferation

The splenocyte proliferation assay was carried out as previously described (Yuan et al., 2010) with some modification. Briefly, 100 μl of splenocyte suspension (5×10^5 cells) was added into 96-well plate, followed by Con A (1.6 μg), LPS (1.6 μg), and PrV antigen (5×10^5 $TCID_{50}$ of heat-inactivated fPrV) or medium to provide 200 μl. The plates were first incubated for 44 h at 37 °C, then MTT method was used and the stimulation index (SI) was evaluated using the formula: SI = OD_{570} value for stimulated cells/OD_{570} value