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Ginsenoside Re as an adjuvant to enhance the immune response to the inactivated rabies virus vaccine in mice

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ABSTRACT

The inactivated rabies virus vaccine (RV) is a relatively expensive vaccine, prone to failure in some cases. Ginsenoside Re (Re) is a saponin isolated from *Panax ginseng*, and has an adjuvant property. Here the adjuvant effect of Re to improve the immune response to the RV is evaluated in mice. ICR mice were immunized with saline, 2.50 mg/kg Re, 20 μl RV, 100 μl RV, or 20 μl of RV adjuvanted with Re (1.25, 2.50 or 5.00 mg/kg). Different time points after boosting, we measured serum antibodies in blood samples and separated splenocytes to detect lymphocyte proliferation and the production of IL-4, IL-10, IL-12, and IFN-γ in vitro. We also compared immunizations containing 20 μl RV and 20 μl RV adjuvanted with Re (5.00 mg/kg) for the expression of $CD4^+$ and $CD8^+$ T-cell subsets at different time points. Results indicated that co-administration of Re significantly enhanced serum antibody titers, increased the $CD4^+$:$CD8^+$ ratio, and enhanced both proliferation responses and IL-4, IL-10, IL-12 and IFN-γ secretions. Both Th1 and Th2 immune responses were activated. The supplementation of the Re (5.00 mg/kg) to 20 μl of RV significantly amplified serum antibody responses and Th1/Th2 responses inducing similar protection as did 100 μl of RV. This suggests that Re could be used to reduce the dose, and therefore the cost, of the RV to achieve the same effective protection. Re merits further studies for use with vaccines of mixed Th1/Th2 immune responses.

1. Introduction

The rabies virus causes a fatal illness characterized by encephalopathy and generalized paresis [1]. It remains a significant threat to human and animal health throughout much of the world [2,3]. Globally, more than 55,000 people die from the infection of the virus each year, with about 99% of those deaths occurring in the developing countries, mainly in Asia and Africa [4,5]. Recent studies conducted in China reported approximately 3000 human deaths from rabies annually, which make China become second only to India worldwide in the number of rabies-related death [6,7].

The domestic dog plays a pivotal role in rabies transmission which accounts for more than 95% of human rabies cases in China [6]. Consequently, the vaccination of dogs against rabies is believed to be one of the most effective approaches for the control of the disease and its transmission to humans [8,9]. Research has shown that the vaccination of 70% of dogs should be sufficient to prevent epidemics and eliminate endemic rabies infections [10]. However, given the fact that there are approximately 200 million dogs in China [6,11] and no definitive dog rabies surveillance system [12], the implementation of such a level of vaccination coverage seems some way off [13].

Most of the commercially available rabies vaccines currently in use for dog prophylaxis in China are inactivated cell-culture vaccines. Compared to many live vaccines, the inactivated rabies vaccine (RV) is generally safe and convenient for use and storage but it suffers from a significant failure rate. Even for dogs that are RV immunized, it is known that the rabies vaccination does not protect humans and animals from rabies in all cases and vaccine failure sometimes occurs where the vaccine is unable to induce a long-lasting protective immune response in some cases. For example, a retrospective study in North America suggested that Alaskan dogs receiving a single injection of RV failed to achieve antibody titers above 0.5 IU/ml in 27% of cases on day 60, 24% on day 180, and 33% on day 360 after the immunization [14]. Minke et al. [15] analyzed serum samples of laboratory dogs having received commercially available RV and found that only 67% of dogs had sufficient antibody titers required for immune protection immunity on day 28. Thereafter the proportion of dogs with protective antibody titers dropped significantly, with only 7% remaining on day 120 after vaccination. Therefore, though increasing dog vaccination coverage rates remains an important future target for China, in the meantime improving the immune effectiveness of rabies vaccines for individually immunized dogs could be a more realistic short term goal to aid in the control rabies transmission between immunized and unimmunized dogs and thereafter to prevent dog-bite acquired rabies in humans.

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In the development of suitable vaccine protocols and antigens, little attention has been paid to the potential of new vaccine adjuvants to enhance the host's immune response. Yet, in most cases, vaccines require the addition of an adjuvant to induce a protective and long-lasting immune response [16]. In addition, rabies vaccines derived from cultured cells are costly to produce and, in some cases, prohibitively expensive to purchase [17]. Our aim is to investigate the addition of Re as an adjuvant with the aim of improving the RV's effect and to examine the potential for Re addition to reduce the required RV immunization dose and thus reduce the cost of vaccination.

The root of *Panax ginseng* C. A. Meyer as a traditional Chinese medicine has been used for at least 2000 years [18], and is believed to be a tonic to stimulate the body resistance against infections [19]. Ginseng saponins (ginsenosides) are believed to be the main pharmacologically active constituents of the plant [19,20]. Ginsenoside Re (Re) is one of the constituents, isolated from the root as well as the stem and leaf of *P. ginseng* [19,20]. Interest in Re for medical and veterinary vaccines has recently increased due to its many advantages, such as ready availability, low cost, high effectiveness, and low risk of side effects and toxicity.

Studies of Re suggest that it possesses a broad range of biological activities. These include antidiabetic effects in obese mice, beneficial effects on cardiac function by suppressing electromechanical alternans (EMA) in rats, as well as significant antioxidant and antihyperlipidemic efficacies in diabetic rats [21,22]. Recent investigations on Re have demonstrated its adjuvant abilities to boost both cellular (Th1) and humoral (Th2) immune responses [23,24]. We previously found that Re also enhanced the immune responses to the model antigen ovalbumin (OVA) [25] and the influenza vaccine (H3N2) in mice [26]. Despite the effects of Re as an adjuvant has been assessed in several vaccines, to the author's knowledge, the immune effects of Re as an adjuvant to the widely used veterinary rabies vaccines have not been investigated. The present study aims to determine whether Re can act as an adjuvant to enhance the immune response to the inactivated rabies virus vaccine in mice.

2. Materials and methods

2.1. Antigen, adjuvant and antibodies

A commercially available rabies vaccine of Rabvac™ 3 Rabies was used in this study (Zoetis Inc., USA). It is a monovalent inactivated rabies vaccine, containing the Street Alabama Dufferin/HCP-SAD strain at ≥1.5 IU/dose ($10^{6.3}$ $FAID_{50}$/ml). The virus had been cultivated in cell line FKCU and was inactivated with beta-propiolactone. Re was extracted from the stem and leaf of *P. ginseng* C. A. Meyer, which had been purchased from Hongjiu Ginseng Industry Co., Ltd. (Jilin, China) as a white powder with 98% purity and a molecular weight of 947. The Re was first dissolved in dimethylsulfoxide (DMSO), diluted with physiological saline solution (1000 μg/ml), and sterilized by passing it through a 0.22 μm filter. The endotoxin level in the above solutions was less than 0.5 endotoxin unit (EU)/ml as determined by a gel-clot Limulus amebocyte lysate assay (Zhanjiang A&C Biological Ltd., Zhanjiang, China). FITC-conjugated anti-mouse CD4 (GK1.5) and anti-mouse CD8a (53-6.7) antibodies, combined with phycoerythrin-conjugated antibodies were purchased from eBioscience (San Diego, CA, USA).

2.2. Mice and immunization

2.2.1. Mice

Female ICR mice (18-22 g) were obtained from Shanghai Laboratory Animal Center and randomly assigned to experimental groups generally consisting of between six to twenty mice in each group. They were housed in polypropylene cages with sawdust bedding in a hygienically controlled environment. The temperature was controlled at 24 ± 1 °C and humidity at 50 ± 10%. Feed and water were supplied *ad libitum*. All procedures related to the animals and their care conformed to the internationally accepted principles as found in the Guidelines for Keeping Experimental Animals issued by the government of China.

2.2.2. Immunization experiment 1

Forty-two ICR mice were randomly distributed into seven groups of six. Each mouse was subcutaneously injected on day 0 and again on day 21 with saline (group 1), 2.50 mg/kg Re (group 2), 100 μl RV (group 3), 20 μl RV (group 4), 20 μl RV adjuvanted with Re of either 1.25 mg/kg (group 5), 2.50 mg/kg (group 6), or 5.00 mg/kg (group 7). Two weeks after boosting, six blood samples from each group were collected to measure the rabies-specific serum antibody (Ab) titers. Splenocytes were prepared to determine the cellular proliferation and production of IL-4, IL-10, IL-12, and IFN-γ.

2.2.3. Immunization experiment 2

Sixty-four ICR mice were randomly distributed into two groups of 32. Each of the animals was subcutaneously immunized on days 0, 7 and 21 with 20 μl of RV (group 1) or 20 μl of RV adjuvanted with Re of 5.00 mg/kg (group 2). At two, four, eight and sixteen weeks after the boost, blood samples from each group ($n = 8$ at each time period), were randomly collected for measurement of rabies-specific serum antibodies titers.

2.2.4. Immunization experiment 3

Forty ICR mice were randomly distributed into two groups of 20. Each of the animals was subcutaneously immunized on day 0, again on day 21 with 20 μl RV (group 1) or 20 μl RV adjuvanted with Re of 5.00 mg/kg (group 2). Two and four weeks after the boost, blood samples from each group ($n = 10$ at each time period) were randomly collected for immunofluorescence analysis of $CD4^+$ and $CD8^+$ T-cell subsets.

2.3. Measurement of serum antibodies

Serum samples were analyzed to measure the rabies-specific antibodies using a commercially available ELISA kit (Zoetis Inc., USA) according to the manufacturer's instructions. Positive and negative control sera were provided in the kit. Briefly, serum samples (1:100) were incubated in plates pre-coated with the rabies virus antigen at 37 °C for 1 h. After four washes, horseradish peroxidase-conjugated protein A was added and incubated at 37 °C for another 1 h. The plate was washed again followed by the addition of TMB substrate. The optical density of the plate was recorded at 450 nm. Sera titers were expressed as equivalent units per ml (EU/ml) corresponding to international units by using the values obtained by the WHO reference serum.

2.4. Lymphocyte proliferation assay

Spleens were collected from the ICR mice under aseptic conditions. They were placed in Hank's balanced salt solution (Sigma), minced, and passed through a fine steel mesh to obtain a homogeneous cell suspension. After centrifugation (380 ×*g* at 4 °C for 10 min), the pelleted cells were washed three times in PBS and resuspended in a complete medium (RPMI 1640 supplemented with 0.05 mM 2-mercaptoethanol, 100 UI/ml penicillin, 100 μg/ml streptomycin, and 10% heat inactivated FCS). Cell numbers were then counted with a hemocytometer by the trypan blue dye exclusion technique. Splenocytes were seeded into a 96-well flat-bottom micro-titer plate at 5.0×10^6 cell/ml in 100 μl of complete medium. Concanavalin A (ConA; final concentration = 5.00 μg/ml), LPS (final concentration = 7.50 μg/ml), or medium was then added to a final volume of 200 μl. The plates were incubated at 37 °C in a humid atmosphere with 5% CO_2 for 2 days. All tests were carried out in triplicate. Cell proliferation was evaluated using the MTT method. In a typical evaluation, 2.5 μl of MTT solution (2 mg/ml) was added to each well 4 h before the end of