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Adjuvant activity of Chinese herbal polysaccharides in inactivated veterinary rabies vaccines

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ABSTRACT

Four botanical polysaccharide preparations (from *Astragalus*, *Echinacea*, wolfberry, and kelp) were evaluated as immunopotentiators/adjuvants of a veterinary rabies vaccine. Results showed that lymphocyte proliferation and some cytokines were significantly elevated, with cellular immune responses skewed towards Th1 and Tc1. All four polysaccharides produced accelerated and enhanced effects on rabies-neutralizing antibody responses in mice and dogs. The results also indicated that certain botanical polysaccharides could be used in rabies vaccine formulations for early and persistent prophylaxis.

1. Introduction

Rabies, a devastating and preventable viral disease, poses a serious threat to humans and animals (especially canid species) in China [1]. More than 95% of the 2000–3000 annual human rabies cases in China are caused by rabid dog bites. This situation is largely the result of the low coverage of rabies immunization in dogs in the country [2–4]. In China, domestic live vaccines (including ERA and Flury-LEP) that are of low potency and with poor safety records in humans and dogs are still used widely [4]. The quality of these vaccines for animal use does not satisfy all of the requirements for an effective vaccine capable of preventing and controlling rabies in China. Although the use of four domestically inactivated rabies dog vaccines has been licensed in the past two years, none has been marketed for several reasons (e.g., potency, cost, etc.). Therefore, improvement of the potency of inactivated vaccines is a basic and necessary requirement to eliminate dog rabies in China fully.

Two methods for enhancing the potency were commonly used in inactivated rabies vaccine in China. One approach, increasing the amount and concentration of viral antigen, is costly and usually performed in human rabies vaccines. Another approach, the addition of immunopotentiators/adjuvants, is generally administered in veterinary biologics.

Polysaccharides derived from different species of flora, including higher plants (such as *Astragalus*, *Isatis* root, and *Epimedium*), microorganisms (including yeasts and mushroom), lichens, and algae, have been proven to boost adaptive immunity through the increase in and/or modulation of innate immunity [5–7]. Interest in these polysaccharides for the development of new adjuvants or immunopotentiators for medical and veterinary vaccines has recently increased due to their many advantages, such as ready availability, low cost, high effectiveness, and low risk of side effects and toxicity [8–11]. Among these polysaccharides, *Astragalus* is one of the most commonly used immune potentiators in domestic animals in China; however, the benefits of incorporation of the polysaccharide into inactivated rabies vaccine have not been demonstrated [12–15]. The effects of *Echinacea*, *Lycium barbarum* (wolfberry), and *Laminaria japonica* (kelp) polysaccharides on enhancing the immune responses have not been reported in dozens of vaccines. In the present study, *Astragalus*, *Echinacea*, wolfberry, and kelp polysaccharides were individually tested to screen for highly effective immunoenhancers/adjuvants for inactivated rabies vaccines.

2. Materials and methods

2.1. Botanical polysaccharides and rabies vaccine

Astragalus (AP, a total polysaccharide containing 80% glucose) and *Echinacea* (EP, a total polysaccharide containing 80% glucose) polysaccharides were obtained from Chengdu Xiya Chemical Technology Co., Ltd. *L. barbarum* (wolfberry) (LBP, a total polysaccharide containing 80% glucose) and the *L. japonica* (kelp) (LJP, a total polysaccharide containing 80% glucose) polysaccharides were

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products of Aladdin Reagent Database Inc., Shanghai, China. The polysaccharides were sterilized by heating at 100 °C for 30 min.

The rabies virus (CVS-11, $10^{7.25}$ $TCID_{50}$/mL) was inactivated using 1/4000 β-propiolactone (BPL, Wako Pure Chemical Industries, Ltd., Japan) at 4 °C for 24 h and then incubated at 37 °C for 2 h to hydrolyze the remaining BPL. Approximately 90 mL of the inactivated rabies virus was mixed with 10 mL of aluminum hydroxide gel (a final concentration of 0.7 mg/mL; Five-star Animal Health Pharmaceutical Factory, Jilin Province, China).

The polysaccharides were added to the rabies vaccine at a final concentration of 2 mg/mL (according to previous studies) [6] for injection in mice or 10 mg/mL for injection in dogs. The vaccine without polysaccharides was used as negative control.

2.2. Virus and cells

BHK-21 cells were routinely maintained in the laboratory under Dulbecco's Minimum Essential Medium (GIBCO) supplemented with 2% newborn calf serum, 100 U/mL of penicillin G, and 100 g/mL of streptomycin and passaged when cells were confluent. The rabies challenge virus standard (CVS-11) was used in the neutralization test to determine the neutralizing antibody titer. The virus titer in BHK-21 cells was $10^{7.25}$ $TCID_{50}$/mL.

2.3. Animals and immunization

Kunming mice (14–16 g) were purchased from the Changchun H&N Animal Breeding Center for Medicine and randomly divided into five groups (50 mice/group), i.e., AP, EP, LJP, LBP, and control. Each mouse was immunized with 0.05 mL of vaccine in hind limb skeletal muscle using a 29G 1/2″ insulin syringe, after which ten random peripheral blood samples were respectively collected from the retro-orbital plexus on days 0, 1, 3, 5, and 14 following vaccination.

Dogs (5–8 kg, 3–4 months old) unimmunized against rabies were obtained from rural areas of Changchun and randomly split into five groups (6 dogs/group). Each dog was inoculated with 1 mL vaccine in the triceps brachii muscle. Peripheral blood samples were collected from the brachial vein before vaccination and every 1 month for 6 months.

All mice and dogs used in the experiments were treated humanely and euthanized by injection of 5% pentobarbital at the end of the experiment.

2.4. Peripheral blood lymphocyte proliferation assay in vitro

Whole blood was collected from the retro-orbital plexus of the non-immune mice at 5 weeks of age and immediately heparinized, then diluted with an equal volume of Hanks' balanced salt solution with Ca^{2+} and Mg^{2+} and carefully layered on the surface of lymphocyte separation medium. After centrifugation at 800 × *g* for 20 min, the cloud-like lymphocyte band was collected and washed twice with RPMI-1640 medium. The resulting pellet was re-suspended at 10^6/mL in RPMI-1640 medium containing 10% FBS and added to 96-well flat-bottomed cell culture plates (100 μL per well). A total of 400 μL of each polysaccharide (AP, EP, LBP or LJP) at 50 μg/mL was added to each of the four wells. An equal volume of 50 μg/mL phytohemagglutinin (PHA; Sigma–Aldrich) was added as the positive control, and cells grown without mitogen were used as negative control. The plates were incubated in a humidified 5% CO_2 incubator in air atmosphere at 37 °C for 48 h. After 44 h, 10 μL of CCK-8 solution (Cell Counting Kit-8, Dojindo Laboratories, Japan) was added to each well. After 4 h, the optical absorbance at a wavelength of 450 nm (OD_{450} value) was measured in each well by a multi-well absorbance reader (Model 680, BIO-RAD) [16].

2.5. Flow cytometry analysis

To discriminate Th1/Th2 and Tc1/Tc2 subsets based on their cytokine expression, whole blood was collected on the 3rd day after vaccination and immediately mixed at a ratio of 1:10 with heparin sodium (130 units/mg, Wako Pure Chemical Industries, Ltd., Japan). Whole blood cells from vaccinated mice were stained with combinations of the following conjugated antibodies (all from eBioscience): Rat IgG1 K isotype Control PE, Anti-Mouse IL-4 PE, Anti-Mouse CD8a FITC, Anti-Mouse IFN gamma PE, and Anti-Mouse CD3e PE-Cy5. The stained cells (10,000 cells) were analyzed using a FACSCalibur™ flow cytometer (3-color from BD Bioscience) [17].

2.6. Cytokine measurement

Some serum cytokine (IFN-α, IL-1β, IL-5, IL-6, and MCP-1) levels related to antibody responses were measured using commercial ELISA kits (Biolegend, Inc., San Diego, USA) according to the manufacturer's instructions.

2.7. Rabies-neutralizing antibody assay (FAVN test)

The FAVN test was performed according to the method described by Cliquet et al. [18]. Briefly, 3-fold serial dilutions of serum samples and controls were prepared in quadruplicate in microplates. Fifty microliters (100 $TCID_{50}$) of challenge rabies virus CVS-11 was added to each well. After 1 h incubation at 37 °C in a humidified 5% CO_2 atmosphere, 50 μL of a BHK-21 cell suspension containing 4×10^5 cells/mL was added to each well and the plates were incubated for 48 h at 37 °C. Following fixation in 80% cold acetone for 30 min at room temperature, the cells were stained by the addition of FITC-conjugated anti-rabies N monoclonal antibody (made in the laboratory) to each well, incubated at 37 °C for 30 min, and then washed with PBS-Tween 20. The plates were examined by fluorescence microscopy (Olympus Corp., Tokyo, Japan) and the presence or absence of fluorescent foci in the cells was recorded. Endpoints were calculated as the inverse of the highest dilution showing no fluorescence, using the Spearman–Kärber formula. A standard anti-rabies serum (AFSSA, Nancy, France) was included as a positive control in all assays, and data were calculated as IU/mL of rabies virus-neutralizing antibodies.

2.8. Statistical analysis

Variance analyses were performed using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA) for Windows to determine statistically significant differences in the generated data by analysis of variance (one-way ANOVA). The results of the comparisons between groups were considered significantly different if $p < 0.05$ and very significantly different if $p < 0.01$. Data are expressed as mean ± SD.

3. Results

3.1. Changes in lymphocyte proliferation

The OD_{450} values of all groups are presented in Table 1, from which lymphocyte proliferation in vaccinated mice was observed to be significantly enhanced in the presence of all the polysaccharides ($p < 0.01$). Responses to the EP, LJP, and LBP groups did not differ significantly, but were lower than those to the AP group.

3.2. Discrimination of Th and Tc subsets in mice by flow cytometry

Th1/Th2 and Tc1/Tc2 subsets of mice were discriminated by detection of the cytokines secreted from $CD3^+$ cells, especially T