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Isatis indigotica root polysaccharides as adjuvants for an inactivated rabies virus vaccine

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ABSTRACT

Adjuvants can enhance vaccine immunogenicity and induce long-term enhancement of immune responses. Thus, adjuvants are important for vaccine research. Polysaccharides isolated from select Chinese herbs have been demonstrated to possess various beneficial functions and excellent adjuvant abilities. In the present study, the polysaccharides IIP-A-1 and IIP-2 were isolated from *Isatis indigotica* root and compared with the common vaccine adjuvant aluminum hydroxide via intramuscular co-administration of inactivated rabies virus rCVS-11-G into mice. Blood was collected to determine virus neutralizing antibody (VNA) titers and B and T lymphocyte activation status. Inguinal lymph node samples were collected and used to measure B lymphocyte proliferation. Splenocytes were isolated, from which antigen-specific cellular immune responses were detected via ELISpot, ELISA and intracellular cytokine staining. The results revealed that both types of polysaccharides induce more rapid changes and higher VNA titers than aluminum hydroxide. Flow cytometry assays revealed that the polysaccharides activated more B lymphocytes in the lymph nodes and more B and T lymphocytes in the blood than aluminum hydroxide. Antigen-specific cellular immune responses showed that IIP-2 strongly induced T lymphocyte proliferation in the spleen and high levels of cytokine secretion from splenocytes, whereas aluminum hydroxide induced proliferation in only a small number of lymphocytes and the secretion of only small quantities of cytokines. Collectively, these data suggest that the polysaccharide IIP-2 exhibits excellent adjuvant activity and can enhance both cellular and humoral immunity.

1. Introduction

Rabies is a fatal form of encephalomyelitis caused by the rabies virus, to which all warm-blooded animals, including humans, are susceptible [1,2]. According to the World Health Organization, approximately 55,000 people die from rabies each year worldwide [3,4]; this total includes more than 3000 people in China [5]. China is second worldwide in terms of the number of rabies-related deaths. Because the mortality rate for rabies is nearly 100% once clinical symptoms appear, the most effective way to prevent the spread of rabies is through inoculation with a vaccine [2]. Previous studies have shown that domestic dogs are responsible for over 95% of human rabies cases [6] and that the immunization of >70% of all domesticated dogs may be sufficient to prevent rabies transmission to humans and avoid a rabies epidemic [7,8].

Most commercial rabies vaccines for human and veterinary use in China are inactivated cell culture vaccines. Inactivated cell culture vaccines are safe and easy to use and store. However, at least 3 injections are required to provide a sufficient virus neutralizing antibody (VNA) titer (at least 0.5 IU/ml), which is a reliable indicator that immune protection against rabies virus infection has been achieved [9,10]. In fact, the rabies neutralizing antibody titer is the most reliable indicator of immune protection [11]. Previous studies have shown that nearly one-third of dogs that received only one injection of a commercial rabies vaccine failed to produce a VNA titer of 0.5 IU/ml [12–15]. The use of multiple injections translates into higher costs and therefore becomes unaffordable for developing countries. Similar circumstances exist for humans. Most human rabies vaccinations are given post-exposure [9], so

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the rapid generation of an antibody response following rabies vaccination is very important. The current post-exposure prophylaxis schedule requires at least 4 injections to be effective, and rabies immune globulin is required in serious cases [10]. Vaccine adjuvants are widely used to accelerate and boost immune responses. Aluminum hydroxide is a commonly used vaccine adjuvant; however, its contribution to early antibody responses is limited, and it may cause side effects in some cases [16–19]. In addition, some studies have shown that aluminum hydroxide may even delay early antibody production [20]. Interestingly, previous records in the Chinese Pharmacopoeia describing the use of aluminum hydroxide in human rabies vaccines were deleted from its fifth edition. Therefore, a novel adjuvant must be developed to increase the effectiveness of the inactivated rabies veterinary vaccine to provide better immune coverage for domestic dogs. This in turn should help block rabies transmission to humans and if approved for human use, could reduce the dosing schedule required for post-exposure prophylaxis.

Recent research has shown that many plant polysaccharides, specifically those derived from Chinese herbs, can enhance immunogenicity and be used to promote both humoral and cellular immunity. These polysaccharides are natural, safe and non-residual [21,22].

The use of *Isatis indigotica* root as a medicine in China can be traced back to the beginning of the Common Era. It is believed that this root can stimulate the body's resistance to influenza and severe acute respiratory syndrome (SARS) and may even prevent these conditions [23–26]. The polysaccharide IIP-A-1 is an alpha-glucan isolated from the roots of *I. indigotica*; its chemical structure and adjuvant activities in influenza H1N1 and hepatitis B surface antigen (HBsAg) vaccines were described in a previous study [27]. IIP-2, another polysaccharide isolated from these same roots, is an arabinogalactan with a molecular weight of 66,400 Da. IIP-2 is composed of arabinose and galactose at a ratio of 1.0:1.5. Its structure and activity as an adjuvant were previously described [28]. Previous studies have also shown that arabinogalactan can be used either to potentiate immune responses or as an adjuvant in human or animal vaccines [29–31].

The present study evaluated the use of the polysaccharides IIP-A-1 and IIP-2 as adjuvants for an inactivated rabies virus vaccine in mice. The effects of antigen-specific humoral and cellular immune responses and their protective capacity when challenged with virulent rabies virus were also analyzed.

2. Materials and methods

2.1. Viruses, cells, polysaccharides and mice

The rabies virus wtCVS-11 was provided by the Chinese Center for Disease Control and Prevention. Recombinant virus rCVS-11-G was recovered and stored in our laboratory, as previously described [32]. A street rabies virus strain, HuNPB3, was isolated from a pig in Hunan Province in 2006 and has been stored in our laboratory. The rCVS-11-G strain was propagated in BSR cells, which were grown in Dulbecco's modified Eagle's minimum essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Baby hamster kidney (BHK-21) cells were grown in DMEM supplemented with 10% FBS. The polysaccharides IIP-A-1 and IIP-2 were kindly provided by Professor Shan Junjie of the Beijing Institute of Pharmacology and Toxicology.

BALB/C mice (6- to 8-week-old females) were purchased from the Changchun Institute of Biological Products (Changchun, China). All animal studies were conducted with prior approval from the Animal Welfare and Ethics Committee of the Military Veterinary Research Institute of the Academy of Military Medical Sciences under permit number SCXK-2014-022. The environment and housing facilities satisfied the National Standards of Laboratory Animal Requirements (GB 14925-2001) of China.

2.2. Immunogen preparation

BSR cells were infected with rCVS-11-G at an MOI of 0.1. The titer of virus collected was 10^8 50% tissue culture infectious dose units ($TCID_{50}$)/ml. rCVS-11-G was inactivated by mixing with 0.03% β-propiolactone; the mixture was incubated overnight at 4 °C and then for 2 h at 37 °C. Inactivated rCVS-11-G was mixed with IIP-A-1 or IIP-2 and incubated overnight at 4 °C. Aluminum hydroxide was mixed completely with inactivated rCVS-11-G at a volumetric ratio of 1:4.

2.3. Mouse immunization and challenge

Mice were randomly divided into 5 groups with 20 mice per group and were inoculated twice with 50 μl of inactivated rCVS-11 (5×10^6 $TCID_{50}$) mixed with different adjuvants at 2-week intervals. A control group was injected twice with 50 μl of inactivated rCVS-11 (5×10^6 $TCID_{50}$) only, and a mock group was injected twice with PBS at the same time points. For each dose, 200 μg of either IIP-A-1 or IIP-2 was included. The mice were challenged with 100× of the 50% intramuscular mouse lethal dose ($IMLD_{50}$) of street rabies virus strain HuNPB3, which was injected into the forelimb muscle 42 days after the first immunization. Following this, the mice were observed for an additional 21 days. During the observation period, all of the mice that developed clinical signs of rabies were humanely euthanized by cervical dislocation under isoflurane anesthesia.

2.4. Antibody response assay

Blood was collected from mice 3, 7, 14, 21, 28 and 42 days after the first immunization by retro-orbital plexus puncture. 6 mice from each group was randomly selected at each time point to represent the mean VNA titers. VNA titers were determined using a fluorescent antibody virus neutralization (FAVN) test [33].

2.5. Interferon-γ and interleukin-4 enzyme-linked immunospot assays

Spleens were collected from 3 mice from each group on day 14 after the second vaccination, and splenocytes were isolated and suspended at a concentration of 1×10^6/ml in RPMI 1640 medium supplemented with 10% FBS. Splenocyte suspensions were stimulated with inactivated HuNPB3 at a concentration of 10 μg/ml. The cell suspensions were then incubated at 37 °C for 24 h. The number of cells which produced interferon (IFN)-γ and interleukin (IL)-4 in the splenocytes was measured using an enzyme-linked immunospot (ELISpot) assay (Mouse IFN-γ and IL-4 ELISPOT kit, Mabtech AB, Stockholm, Sweden) according to the manufacturer's instructions. The number of spot-forming cells (SFCs) was determined using an automated ELISpot reader (AID GmbH, Strasberg, Germany).

2.6. Flow cytometry assays to assess intracellular cytokine staining

Splenocytes were isolated from 3 mice from each group at 14 days after the second immunization, and splenocyte suspensions (1×10^6 cells/ml) were prepared in RPMI 1640 medium containing 10% FBS. The splenocyte suspensions were stimulated with inactivated HuNPB3 at a concentration of 10 μg/ml and were cultured with a protein transport inhibitor (containing monensin) (BD Biosciences, Franklin, TN, USA) at 37 °C. The cell suspensions were