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

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** Article Info **

Received 9 December 2015
Received in revised form 2 February 2016
Accepted 8 February 2016
Available online 10 February 2016

Keywords:
Rabies vaccine
Adjuvant
Antibody titer
Cellular immunity
Polysaccharide

** 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.

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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
the rapid generation of an antibody response following rabies vac-
cination 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 adju-
ivants are widely used to accelerate and boost immune responses.
Aluminum hydroxide is a commonly used vaccine adjuvant; how-
ever, 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 delay early
antibody production [20]. Interestingly, previous records in the
Chinese Pharmacopoeia describing the use of aluminum hydro-
oxide 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 polysaccha-
drides, 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 com-
posed of arabinose and galactose at a ratio of 1:0.1:1.5. 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 vir-
ulent 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 labo-
ratory. The rCVS-11-G strain was propagated in BSR cells, which
were grown in Dulbecco's modified Eagle's minimal essential
medium (DMEM) supplemented with 10% fetal bovine serum (FBS).
Baby hamster kidney (BHK-21) cells were grown in DMEM supple-
mented 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 hous-
ing 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 $10^6$ 50% tissue culture infectious dose
units (TCID$_{50}$)/ml. rCVS-11-G was inactivated by mixing with 0.03%
$\beta$-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$^4$ TCID$_{50}$) mixed with different adjuvants at 2-week inter-
valls. A control group was injected twice with 50 μl of inactivated
rCVS-11 (5 × 10$^4$ 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 (MLD$_{50}$) 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 rep-
resent 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 $1 \times 10^6$/ml in RPMI 1640 medium
supplemented with 10% FBS. Splenocyte suspensions were stim-
ulated 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 deter-
mined 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 inac-
тивated HuNPB3 at a concentration of 10 μg/ml and were cultured
with a protein transport inhibitor (containing monensin) (BD Bio-
sciences, Franklin, TN, USA) at 37°C. The cell suspensions were
Results were isolated, each CD19 and anti-mouse IL-4 antibodies (BD Biosciences) for 30 min at 4°C. The stained cells were analyzed using a flow cytometer.

2.7. Flow cytometry assays to analyze B and T cell populations in lymph nodes and blood collected from mice

Inguinal lymph node samples were harvested from 3 mice from each group at 3 and 6 days after the first immunization. Cells were isolated, and single-cell suspensions were prepared in phosphate-buffered saline (PBS) and stained for 30 min at 4°C with CD19 and CD40 antibodies (BD Bioscience) to label B cells. The stained cells were washed twice with PBS and analyzed using a flow cytometer.

Whole-blood samples were collected from 3 mice from each group at 6 days after primary immunization by retro-orbital plexus puncture. Peripheral blood mononuclear cells (PBMCs) were isolated, and single-cell suspensions (1 x 10⁶ cells/ml) were prepared in PBS. The cells were stained for 30 min at 4°C with anti-mouse CD19 and CD40 (BD Biosciences) to label B cells and CD3, CD4 and CD8 monoclonal antibodies (BD Biosciences) to label T cells. The labeled cells were washed twice with PBS and analyzed using a flow cytometer.

2.8. IL-2, IL-4, IL-10 and IFN-γ enzyme-linked immunosorbent assays

Splenocytes were isolated from 6 mice from each group at 28 days after the first vaccination and then suspended in RPMI 1640 medium containing 10% FBS at a concentration of 2 x 10⁶ cells/ml. The splenocyte suspensions were stimulated with inactivated HuNPB3 at a final concentration of 10 µg/ml and then incubated at 37°C. The supernatants from the suspensions were collected at 48h post-stimulation and measured using an enzyme-linked immunosorbent assay (ELISA) (Mouse IL-2, IL-4, IL-10 and IFN-γ ELISA kit, Mabtech AB) according to the manufacturer’s instructions.

2.9. Post-exposure immune test in mice

Mice that were divided into 4 groups with 10 mice per group and were challenged with 10^5 MLD₉₀ of street rabies virus strain HuNPB3 in the muscle of the forelimb. Twenty-four hours after the challenge, the mice were immunized with 10⁷ TCID₅₀ of rCVS-11-G mixed with different adjuvants (alhydrogel, IIP-A-1 or IIP-2); the mock group was injected with PBS. Each group was immunized twice at 24 and 96h after the challenge. The mice were observed for 21 days, and all mice that developed clinical signs of rabies during the observation period were humanely euthanized by cervical dislocation under isoflurane anesthesia.

2.10. Statistical analysis

Data are expressed as the means ± standard deviations (SDs). Statistical analyses were performed using SPSS 13.0 software (SPSS Inc., Chicago, IL, USA) to determine statistically significant differences in the generated data by one-way analysis of variance (ANOVA). Statistically significant differences in survivor ratios were determined by Kaplan–Meier analysis. The results were considered significant if p < 0.05 and very significant if p < 0.01.

3. Results

3.1. Enhancing effects of polysaccharides on rabies neutralizing antibody titers in mice

Blood was collected to determine antibody titers at predetermined times after immunization. Fig. 1 shows the mean VNA titers against rabies virus in mice immunized with rCVS-11-G mixed with different adjuvants. None of the antibody titers rose above 0.5 IU/ml by 3 days after the first immunization, but the titers in the mice immunized with the vaccine in addition to IIP-2 rose to 0.56 IU/ml by 7 days after the first immunization. The mean VNA titer of all four groups rose above 0.5 IU/ml by 14 days after the first immunization; the second immunization was done after we collected the blood at the fourteenth day after the primary immunization. The antibody titers in the mice in the two polysaccharide test groups were higher than those in the alhydrogel group. The VNA titers in the mice from the mock group did not rise above 0.02 IU/ml (data not shown and Fig. 1).

3.2. Antigen-specific cellular immune responses induced by the addition of polysaccharides

After confirming that the studied polysaccharides could enhance VNA responses in mice, we used an ELISPot assay to detect antigen-specific IFN-γ and IL-4 activities in splenocytes. As shown
Fig. 2. ELISpot analysis of IFN-γ and IL-4 secretion and ICS assays for antigen-specific CD4+ and CD8+ T cell secretion of IFN-γ and IL-4 in mouse splenocytes. Spleens were collected from 3 mice per group 14 days after the second vaccination, and splenocytes were assayed by ELISpot and ICS assays. SFCs secreting IFN-γ (A) and IL-4 (B) were measured using a commercial ELISpot kit. RABV-specific CD4+ and CD8+ T cells were measured via ICS assays. Spleens were collected from 3 mice per group 14 days after the second vaccination and were stained with mouse anti-CD4, anti-CD8, anti-IFN-γ and anti-IL-4 monoclonal antibodies. CD4+ cells secreting IFN-γ (C) or IL-4 (D) and CD8+ cells secreting IL-4 (E) or IFN-γ (F) are shown in Fig. 3. The data represent the subtraction value means and SDs of 3 mice and were analyzed using one-way ANOVA (*p < 0.05, **p < 0.01, ***p < 0.001).

in Fig. 2A and B, there were greater numbers of SFCs in the mice injected with IIP-2 than in the mice from the other groups.

We also analyzed the capacities of these adjuvants to induce IFN-γ and IL-4 secretion from CD4+ and CD8+ T cells by intracellular cytokine staining (ICS). As shown in Fig. 2D and F, IIP-2 induced greater numbers of IFN-γ-secreting CD4+ and CD8+ T cells than the other adjuvants tested; similar results were also observed for IL-4-secreting CD4+ and CD8+ T cells (Fig. 2C and E). The results from the other groups showed no significant differences.

3.3. Enhancing effects of polysaccharides on B cell activation in lymph nodes

To investigate whether polysaccharides can function as adjuvants to further induce B cell activation, lymph nodes were collected from the mice. Cells were isolated from the collected lymph nodes and analyzed by flow cytometry. As shown in Fig. 3A and B, the numbers of activated B cells (CD19+CD40+) in the lymph nodes were significantly higher in the IIP-A-1 and IIP-2 groups than
in the alhydrogel group at days 3 and 6 after the first immunization. The representative scatter plots of B cells (CD19⁺CD40⁺) in the lymph nodes are shown in Fig. S1(A and B).

3.4. Enhancing effect of polysaccharides on B and T cell recruitment in the blood

To further investigate the use of polysaccharides as adjuvants, blood was collected, and PBMCs were isolated, cultured, and stained as described above. The resultant cell suspensions were analyzed by flow cytometry. Fig. 4A and B shows T cell recruitment (CD3⁺CD4⁺, CD3⁺CD8⁺), and Fig. 4C shows B cell activation (CD19⁺CD40⁺) in blood. IIP-2 activated the greatest numbers of B and T cells among the four tested adjuvant conditions. Fig. S2(A and B) shows the representative scatter plots of T cell (CD3⁺CD4⁺, CD3⁺CD8⁺) and B cell (CD19⁺CD40⁺) in blood.

3.5. Enhancing effects of polysaccharides on splenocyte cytokine secretion

Levels of the cytokines IL-2, IL-4, IL-10 and IFN-γ, all of which are secreted by splenocytes, were measured using commercial ELISA kits. As shown in Fig. 5A and D, the levels of IL-2 and IFN-γ secreted from splenocytes isolated from mice in the IIP-A-1 and IIP-2 groups were significantly higher than those detected in the other experimental groups. In Fig. 5B and C, the levels of IL-4 and IL-10 in all polysaccharide groups were higher than those in the mock and alhydrogel groups.

3.6. Immunization with polysaccharide adjuvants protects mice against lethal challenge with a street rabies virus strain

To evaluate whether the immune responses induced by the administration of inactivated rCVS-11-G mixed with polysaccharides could provide protection against a rabies infection, mice were challenged with street rabies virus strain HuNPB3. All mice from the polysaccharide groups survived, whereas one mouse from the aluminum hydroxide group and three mice from the control group did not. None of the mice from the PBS group survived. The results of the challenge test are shown in Fig. 6A. Clinical symptoms and RT-PCR results produced by assessing total RNA from brain tissues collected from the dead mice revealed that the mice died from rabies infection (data not shown). The results of the challenge test suggest that IIP-A-1 and IIP-2 can provide complete protection against street rabies virus infection when mixed with an inactivated rabies vaccine.

To determine the protective effects of polysaccharides as adjuvants in post-exposure prophylaxis, mice were challenged with HunPB3 24 h before immunization. Only one mouse from the aluminum hydroxide group survived the challenge. In contrast, 3 mice from the IIP-A-1 group and 7 mice from the IIP-2 group survived for the duration of the observation period, whereas all mice in the mock group died (Fig. 6B). Clinical symptoms and RT-PCR results produced by assessing total RNA isolated from brain tissues collected from the dead mice indicated that the mice died from rabies infection (data not shown).
4. Discussion

Due to safety concerns, all human rabies vaccines in use today are inactivated virus vaccines. Although these vaccines are safe, they induce only mild immune responses and therefore require multiple injections to generate antibody production, which proceeds at a slow rate [10]. Post-exposure immunization is the main immunization method used in human. The incubation period for rabies can be as short as one week, and rabies immune globulin is needed in severe cases [1]. The recommended dose for equine rabies immune globulin is 40 IU/kg [34], which translates into a need for 2000 IU to 3000 IU of equine immune globulin per individual. In other words, at least 2 vials or doses of rabies immune globulin are required per case. The cost of such a treatment regime is barely affordable for the inhabitants of developing countries. The cost for one dose of equine rabies immune globulin in Cambodia, a developing country in Southeast Asia, ranges from US$20 to US$35. This is far too expensive for the farmers living in the rural areas of Cambodia, whose monthly income is typically less than US$80 [35]. Although the dose recommended for human rabies immune globulin (HRIG) is only 20 IU/kg, the cost of HRIG is even more expensive. Post-exposure prophylaxis may also fail if insufficient immune globulin is used or if the immune globulin is used improperly [34,36]. Such scenarios occur in developing countries because of their lack of skilled physicians. Thus, because of the high cost of rabies immune globulin, improving the antibody production rate is critical for generating a post-exposure rabies vaccine that is effective for human use, especially in developing countries. An adjuvant that can accelerate the generation of antibody responses and induce cellular immunity would be optimal. Aluminum hydroxide, a traditional, widely used adjuvant, stimulates the immune response by inducing Th2 responses, prolonging the exposure of an antigen to the immune system and delaying antigen clearance from an immunization site [16,17,37]. These mechanisms contribute only slightly to early antibody responses and cellular immunity. Furthermore, side effects, such as severe inflammatory and nervous system concerns, may appear in certain cases. Polysaccharides are emerging as a new type of adjuvant that can be used to stimulate a rapid immune response, thereby serving as a highly valuable vaccine addition.

Although β-glucan is the most well-known immune response stimulant [38], a few studies have reported that α-glucan can also be used as an adjuvant for the same purpose in human and veterinary vaccines. RR1, an (1 → 4)-α-D-glucan with a molecular weight of 550 kDa that was isolated from Tinospora cordifolia, has been shown to activate lymphocytes in vitro and stimulate their secretion of IL-1β, IL-6, IL-12, IL-18, TNF-α, and tumor necrosis factor (TNF)-α, but not their secretion of IL-2, IL-4 or IL-10 [39]. IIP-A-1 is a (1 → 4)-α-glucan with a molecular weight of 3600 Da. IIP-A-1 was evaluated in the current study for its capacity to serve as an immune stimulator. To accomplish this, splenocytes were isolated and cultured in vitro, and the levels of secreted IL-2, IL-4 and IL-10 were assessed; in all three cases, secretion was significantly enhanced (IL-2, p = 0.006; IL-4, p = 0.000; and IL-10, p = 0.036). We also measured rabies-specific VNA titer, the most important gauge of the effectiveness of a rabies vaccine. We found that mice that received IIP-A-1 as a vaccine adjuvant had significantly higher VNA
titers against the rabies virus than did mice that received aluminum hydroxide on days 21 and 28 post-immunization (p_{21} = 0.003 and p_{28} = 0.045). Cellular immunity also plays an important role in resistance to rabies infection and may clear the rabies virus from the CNS [40]. Th1 cells, which are derived from CD4 cells, secrete type 1 cytokines, such as IL-2 and IFN-γ, which possess antiviral activities and stimulate CD8+ cell proliferation [41,42]. Additionally, Th2 cells, also derived from CD4 cells, secrete type 2 cytokines, such as IL-4 and IL-10, which induce helper activity in B cells and stimulate the humoral immune response [43]. These cytokine profiles indicate that RR1 mediates immunity via the Th1 pathway, while IIP-A-1 mediates immunity via the Th2 pathway.

Arabinogalactans are highly branched polysaccharides that can easily dissolve in water; these molecules have high molecular weights and consist of l-arabinose and d-galactose moieties [44]. Several studies have shown that the adjuvant activities of arabinogalactans largely depend on their molecular weights, chemical structures and polysaccharide chain conformations [45,46]. ResistAid™, an arabinogalactan product isolated from the larch plant with a highly branched structure and a 1→3,6-β-d-galactan backbone, is composed of arabinose and galactose units in a volumetric ratio of 6:1. Its adjuvant qualities in relation to IgG production were tested orally in a double-blind parallel-group study evaluating the 23-valent pneumococcal vaccine. No signs of toxicity or side effects were observed when ResistAid™ was used at a dose of 4.5 g/day for 10 weeks in healthy volunteers [30]. Larch plant arabinogalactans also appear to have low toxicity, as no side effects or signs of toxicity were apparent in either mice or rats when they were administered at a dose of either 5 g/kg or 500 mg/kg for 3 months [47]. In the current study, another arabinogalactan, IIP-2, which was isolated from the roots of I. indigotica, was evaluated. IIP-2 possesses a 1→3,6-β-d-galactan backbone similar to that found in larch arabinogalactans. Its adjuvant qualities were assessed using the inactive rabies virus rCIVS-11-G. We first measured the rabies-specific antibody response, the most important indicator of vaccine efficacy. The results of an FAVN test showed that mice treated with IIP-2 had significantly higher VNA titers than mice treated with aluminum hydroxide after a second vaccination (p_{21} = 0.000, p_{28} = 0.009, and p_{42} = 0.036). A few studies have reported that arabinogalactans can enhance the effectiveness of cellular immune responses. For example, G1-4α, an arabinogalactan isolated from T. cordifolia that has a chemical structure similar to IIP-2, was shown to activate bone marrow-derived dendritic cells (BMDCs) in vitro and spleen dendritic cells in vivo. Secretion of the cytokines IL-12 and TNF-α from BMDCs and T cell stimulation also increased [29]. In the current study, we evaluated antigen-specific cellular immune responses by ELISpot and ICS. Our results indicate that IIP-2 can activate spleen cells and stimulate them to secrete IL-2, IL-4, IL-10 and IFN-γ. These findings suggest that IIP-2 can mediate immune responses through both the Th1 and Th2 pathways. Flow cytometry analysis showed that mice treated with IIP-2 had higher numbers of activated T and B lymphocytes in their blood and lymph nodes than

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**Fig. 5.** ELISA results showing the quantities of IL-2, IL-4, IL-10 and IFN-γ secreted by splenocytes. Spleens were isolated from 6 mice per group 14 days after the final vaccination, and isolated splenocytes were cultured and stained as described in Section 2. IL-2 (A), IL-4 (B), IL-10 (C) and IFN-γ (D) levels were measured using a commercial ELISA kit. Representative data are shown as the means ± SDs of 6 mice per group and were analyzed using one-way ANOVA (*p < 0.05, **p < 0.01, ***p < 0.001).
control mice. The results of challenge and post-exposure immune tests showed that IIP-2 mixed with rCSVs-11-G could protect mice from a challenge with a lethal street rabies virus strain.

Both of the polysaccharides analyzed in the current study, particularly IIP-2, enhanced antigen-specific cellular immune responses in spleen cells by activating T cells and promoting cytokine secretion. In contrast, aluminum hydroxide did not significantly affect either spleen cells or lymphocytes. Together, these results suggest that the polysaccharides evaluated here can significantly enhance cellular immune responses and accelerate antibody responses to an inactivated rabies vaccine. Enhanced B and T lymphocyte proliferation and increased cytokine secretion may be the principal mechanisms by which this enhancement is achieved.

Aluminum hydroxide could only induce a slow antibody response that included the secretion of only a few cytokines. Thus, our results suggest that plant root polysaccharides may be more effective for improving antibody responses than traditional adjuvants when used in conjunction with an inactivated rabies vaccine. This increased effectiveness accelerates immune protection and reduces the required vaccine dosage. Overall, our results indicated that both of the polysaccharides tested in this study, in particular IIP-2, can be used as adjuvants in conjunction with an inactivated rabies vaccine to induce robust immune responses in animals and potentially in humans. In conclusion, our work highlights the potential of using polysaccharides to develop a more affordable post-exposure vaccine for use in humans.

**Conflict of interest**

The authors declare no conflicts of interest.

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**Acknowledgements**

This work was supported by the Public Welfare (Agricultural) Industry Research Special Program (Grant Nos. 201103032 and 201303042) and the National Natural Science Funds of China (Grant No. 31402175).

**Appendix A. Supplementary data**

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jibiomac.2016.02.023.

**References**

[1] B. Dietzschold, M. Schnell, H. Koprowski, Pathogenesis of rabies, Curr. Top. Microbiol. Immunol. 292 (2005) 45–56.
[2] Z.F. Fu, Rabies and rabies research: past, present and future, Vaccine 15 (Suppl) (1997) S20–S24.
[3] D.L. Knobel, S. Cleaveland, P.G. Coleman, E.M. Fèvre, M.I. Meltzer, M.E. Miranda, A. Shaw, J. Zinsstag, F.X. Meslin, Reevaluating the burden of rabies in Africa and Asia, Bull. World Health Organ. 83 (2005) 360–368.
[4] World Health Organization, WHO Expert Consultation on Rabies, Second Report, World Health Organ. Tech. Rep. Ser., 2013, pp. 1–139 (back cover).
[5] C.E. Rupprecht, A tale of two worlds: public health management decisions in human rabies prevention, Clin. Infect. Dis. 39 (2004) 281–283.
[6] B.L. Hu, A.R. Fooks, S.F. Zhang, Y. Liu, F. Zhang, Inferior rabies vaccine quality and low immunization coverage in dogs (Canis familiaris) in China, Epidemiol. Vet. Infect. 136 (2008) 1556–1563.
[7] S.L. Davlin, H.M. Vonvile, Canine rabies vaccination and domestic dog population characteristics in the developing world: a systematic review, Vaccine 30 (2012) 3492–3502.
[8] K.M. Charlton, Report of the Meeting Control of Rabies in the Americas, Animal Diseases Research Institute, 11–13 September 1991, Can. Vet. J. 33 (1992) 98–99.
[9] R.A. Nguyen, T.T. Nguyen, D.V. Nguyen, G.C. Ngo, C.N. Nguyen, K. Yamada, K. Noguchi, K. Ahmed, H.D. Hoang, A. Nishizono, Evaluation of rapid neutralizing antibody detection test against rabies virus in human sera, Trop. Med. Health 43 (2015) 111–116.
[10] M.K. Mittal, Revised 4-dose vaccine schedule as part of postexposure prophylaxis to prevent human rabies, Pediatr. Emerg. Care 29 (2013) 1119–1121 (quiz 1122–1114).
[11] A. Ondrejkova, J. Suli, R. Ondrejka, Z. Benisek, R. Franka, S. Scrveck, M. Madar, A. Bugarsky, Comparison of the detection and quantification of the rabies antibodies in canine sera, Vet. Med. Czech 47 (2002) 218–221.
[12] T. Takayama, Anti-rabies antibody levels observed in subjects who were bitten by supposed rabid animals: abroad and received post-exposure immunization, Kansenshogakai Zasshi 72 (1998) 1046–1049.
[13] G. Sage, P. Khawplod, H. Wilde, C. Lobag, T. Hemachudha, W. Tepsumethanon, B. Lumlertdaecha, Immune response to rabies vaccine in Asian dogs: failure to achieve a consistently protective antibody response, Trans. Soc. Roy. Med. Hyg. 87 (1993) 593–595.
[14] J.M. Minke, J. Bouvet, F. Cliquet, M. Wasniewski, A.L. Guiot, L. Lemaître, C. Carieu, V. Cozet, L. Vergne, F.M. Guigal, Comparison of antibody responses after vaccination with two inactivated rabies vaccines, Vet. Microbiol. 133 (2009) 283–286.
[15] S. Van De Zande, M. Kaashoek, W. Hesseling, D. Sutton, T. Nell, Comments to Comparison of antibody responses after vaccination with two inactivated rabies vaccines [Minke, J.M. et al., 2009]. Vet. Microbiol. 133, 283–286, Vet. Microbiol. 138 (2009) 202–203.
[16] E.B. Lindblad, Aluminum compounds for use in vaccines, Immunol. Cell Biol. 81 (2004) 497–505.
[17] C. Exley, P. Siesjo, H. Eriksson, The immunobiology of aluminium adjuvants: how do they really work? Trends Immunol. 31 (2010) 103–109.
[18] E.B. Lindblad, Aluminum adjuvants—in retrospect and prospect, Vaccine 22 (2004) 3658–3668.
[19] M.A. Aprile, A.C. Wardlaw, Aluminum compounds as adjuvants for vaccines and toxoids in man: a review, Can. J. Public Health 57 (1966) 343–360.
[20] P.P. Lin Haixiang, Influence of aluminum adjuvant to experimental rabies vaccine, Chin. J. Exp. Clin. Virol. 13 (1999) 133–136.
[21] Y. Liu, S. Zhang, F. Zhang, R. Hu, Adjuvant activity of Chinese herbal polysaccharides in inactivated veterinary rabies vaccines, Int. J. Biol. Macromol. 50 (2012) 598–602.
[22] X. Su, Z. Pei, S. Hu, Ginsenoside Re as an adjuvant to enhance the immune response to the inactivated rabies virus vaccine in mice, Int. Immunopharmacol. 20 (2014) 283–289.
[23] X.L. Wang, M.H. Chen, F. Wang, P.B. Bu, S. Lin, C.G. Zhu, Y.H. Li, J.D. Jiang, J.G. Shi, Chemical constituents from root of Isatis indigotica, Zhongguo Zhong Yao Za Zhi 38 (2013) 1172–1182.
