Adjuvant activities of seven natural polysaccharides on immune responses to ovalbumin in mice

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Abstract: **OBJECTIVE** To find good adjuvant candidates from natural polysaccharides. **METHODS** Astragalus polysaccharide (APS), Angelica root polysaccharides (ARPS), Epimedium polysaccharides (EPS), Isatis root polysaccharide (IRPS), Lycium polysaccharides (LCPS), Poria polysaccharides (PPS) and Solomonseal polysaccharides (SPS) were extracted, respectively. The carbohydrate contents were determined by phenol-sulfuric acid method. The uronic acids contents of these polysaccharides were measured by *m*-hydroxylbiphenyl assay. The molecular mass characters were identified by gel permeation chromatography. BALB/c mice were intramuscularly injected with ovalbumin (OVA) 60 μg, OVA 60 μg mixed with aluminum hydroxide (0.1 mg) or polysaccharide (1 mg) respectively on day 1, 28 and 56. Serum samples were collected on day 21, 52 and 70 for measurement of anti-OVA special antibody. **RESULTS** The carbohydrate contents (%) were 65.41 for IRPS; 30.88 for ARPS; 43.70 for APS; 48.88 for SPS; 58.68 for PPS; 45.83 for LCPS; 32.60 for EPS. The uronic acids contents (%) were 13.36 for IRPS; 19.73 for ARPS; 6.53 for APS; 5.96 for SPS; 1.96 for PPS; 8.96 for LCPS; 7.53 for EPS. The results of molecular mass showed different distritutiion characters among these polysaccharides. The initial immunity with seven polysaccharides and alum did not elicit significant immune responses against OVA. After the second immunization, only EPS induced a high level of OVA-specific antibody with the titer of $1:10^5$. After the third time of immunization, the antibody titers induced by EPS were further increased, and the anti-OVA antibody titers of mice treated with IRPS, ARPS and PPS reached $1:10^5$. The specific antibody levels induced by four polysaccharides were significantly different from those of alum groups and no adjuvant control. **CONCLUSION** Four polysaccharides, IRPS, ARPS, PPS and EPS, exhibited good adjuvant activities. EPS showed strong potential to increase humoral immune responses. They could be efficacious candidates for new adjuvants.

Key words: polysaccharides, *Angelica sinensis*; polysaccharides, *Astragalus membranaceus*; polysaccharides, *Isatis indigotica*; polysaccharides, *Poria cocos*; polysaccharides, *Polygonatum sibiricum*; polysaccharides, *Lycium barbarum*; polysaccharides, *Epimedium koreanum*; immune; adjuvant

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Adjuvants are compounds that can increase and/or modulate the intrinsic immunogenicity of an antigen and elicit strong and long lasting immune responses. Adjuvants are very important for purified, subunit and synthetic vaccines with poorly immunogenicity[1]. Based on the primary mechanism of action, adjuvants are generally divided into two categories: delivery systems and immune potentiators. So far, only aluminum-based delivery system adjuvants have been approved for human use in prophylactic vaccines, and no immune potentiator adjuvants have been licensed[1-2]. However, alum adjuvant often causes undesirable side-effects, such as sterile abscesses, eosinophilia, myofascitis and granuloma[3-4]. In addition, there was evidence that aluminum compounds might accelerate the incidence of Alzheimer disease[5-6].

With the development of vaccine technology, a number of adjuvants capable of inducing clinically important immune responses have emerged. Last year, promising candidates were discovered that may finally adjunct or displace aluminum sub-

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stances as the main adjuvant. Animal experiments and modern clinical trails have shown that polysaccharides from some traditional Chinese medicines are immunologically active and excellent immunomodulating agents[7]. Most polysaccharides derived from these plants are relatively nontoxic and unlikely to cause significant side effects[8-9]. Recently, many polysaccharides have been shown to not only possess adjuvant potential on specific cellular and humoral immune responses against antigens, but serve as be excellent candidates to replace alum as the adjuvant for many vaccines[10-12]. Used as adjuvants to induce cellular and humoral immunity, plant polysaccharides offer excellent safety, tolerability, and easy to isolate and purify. Thus, these polysaccharide adjuvants have enormous potential for use in vaccines against both pathogens and cancer[13-14].

To find a new-generation vaccine adjuvant, seven natural polysaccharides from traditional Chinese medicine were isolated. They were *Astragalus* polysaccharide (APS), *Angelica* root polysaccharides (ARPS), *Isatis* root polysaccharide (IRPS), *Epimedium* polysaccharides (EPS), *Poria* polysaccharides (PPS), *Lycium* polysaccharides (LCPS) and *Solomonseal* polysaccharides (SPS), respectively. Their adjuvant activities were evaluated using OVA as an antigen in a time-course study compared with alum and no adjuvant control. Being an indicator of humoral immunity, the titers of serum antibody in the mice that received OVA and polysaccharides were measured.

1 MATERIALS AND METHODS

1.1 Experimental animal

Female BALB/c mice (SCXK 2007-004, GradeⅡ, 4-6 weeks old, 18~22 g) were bred in the Experimental Animal Center of the Academy of Military Medical Sciences, Beijing. The mice were housed under standard environment conditions (22-25℃, humidity 50%-70%, 12 h light: 12 h dark cycle) with free access to standard diet and water ad libitum. All animal procedures were performed according to the Guide for the Care and Use of Laboratory Animal of the National Institute of Health as well as Guide of the Animal Welfare Act.

1.2 Herbs and equipments

The seven Chinese medicines were purchased in Tong-Ren-Tang Pharmacy of Beijing in China. They were identified by Prof. MA Qi-yun, Institute of Pharmacology and Toxicology. The roots of *Angelica sinensis* and *Astragalus membranaceus* were produced in Gansu province, the roots of *Isatis indigotica* and the fungus of *Poria cocos* were from Anhui province, the roots of *Polygonatum sibiricum* were from Beijing, the fruits of *Lycium barbarum* were from Ningxia, the leaves of *Epimedium koreanum* were from Jilin province in China. Aluminum hydroxide was purchased from Sinopharm Chemical Reagent Co., LTD. HRP-GAM-IgG kit was purchased from Beijing Zhongshan Golden Bridge Biotechnology Co., LTD. micro-ELISA Reader was Thermo Scientific VARIOSKAN FLASH(USA).

1.3 Extraction and isolation of seven polysaccharides

The leaves of *Epimedium koreanum* (1 kg) were first socked with 10 L petroleum ether (BP 60-90℃) at room temperature for 24 h to remove chlorophyll and terpenes, then filtrated before the residues were dried in the air. The residual leaves were socked with 12 L distilled water at room temperature for 36 h, then filtrated and centrifugated. The residues were treated again under the same condition. The combined supernatant was concentrated to 1 L by evaporation under reduced pressure at 50℃, and 3 L of 95% ethanol was added to precipitate total polysaccharides for 48 h. The precipitated materials were collected by centrifugation, dissolved with distilled water, and dialyzed against top water for 48 h, and distilled water for 24 h at room temperature. The dialyzed liquid was centrifugated, concentrated and freeze-dried to obtain a brown polysaccharides (named EPS, yield 0.51%).

The fruits of *Lycium barbarum* (1 kg) were pulverized and extracted with 8 L of 95% ethanol at room temperature for 48 h to remove lipid and pigments. The supernatant was removed, and the residue was extracted with 12 L distilled water at 50℃ for 4 h. After centrifugation, the residue of fruits was extracted again with the same treatment. The combined water extract was concentrated to 1 L by evaporation under reduced pressure at 50℃, and 3 L of 95% ethanol was added to precipitate total polysaccharides for 48 h. Then the precipitated polysaccharide were dissolved in water, centrifuged, dialyzed against water and freeze-dried to obtain a yellow polysaccharides

(named LBPS, yield 0.82%).

The same method was used to extract total polysaccharides from *Polygonatum sibiricum*. This operation obtained a brown polysaccharide (named SPS, yield 3.16%).

The roots of *Astragalus membranaceus* (1 kg) were pulverized and extracted with 12 L distilled water at 50℃ for 4 h. After centrifugation, the residue of roots was extracted again under the same condition. The combined supernatant was concentrated to 1 L, and 3 L of 95% ethanol was added to precipitate total polysaccharides for 48 h. Then the precipitated polysaccharides were dissolved, centrifuged, dialyzed and freeze-dried to obtain a white polysaccharide (named APS, yield 0.96%).

The total polysaccharides of *Angelica sinensis*, *Isatis indigotica* or *Poria cocos* were extracted and obtained respectively with the same method as *Astragalus membranaceus*. The polysaccharide of *Angelica sinensis* named ARPS was white with a yield of 1.68%. The polysaccharides of *Isatis indigotica* named IRPS was ivory with a yield of 0.42%. The polysaccharides of *Poria cocos*, named PPS, with the yield of 0.16%.

1.4 Characterization of the seven polysaccharides

Total carbohydrate contents of seven polysaccharides were measured by the phenol-sulfuric acid method using glucose as the standard[15]. Uronic acid contents of these polysaccharides were determined by *m*-hydroxylbiphenyl method with minor modification using glucuronic acid as the standard[16-17]. The molecular weights of these polysaccharides were determined by the gel permeation chromatography (GPC) using TSK-GELG4000SW$_{XL}$ column and RID detector. The mobile phase was Na_2SO_4 0.1 mol·L^{-1} and the flow rate was 0.6 ml·min^{-1}. A standard curve of molecular mass with different molecular weights of dextrans was established.

1.5 Immunization and measurement of OVA-specific antibody

Total 50 BALB/c mice were divided into ten groups. Each contained five mice. Animals were immunized by intramuscular injection with OVA 60 μg alone or with OVA 60μg dissolved in saline contained alum (0.1 mg each mouse) or different polysaccharides (1 mg each mouse) respectively on d 1. Immunization was performed three times at a 28 d-interval and mice were sacrificed 14 d after the third immunization. Serum samples collected from mice on d 21, d 52 and d 70 were used for measurement of anti-OVA antibody.

1.6 Enzyme immunoassay for the detection of anti-OVA antibody in serum

The anti-OVA antibody in serum samples was analyzed by ELISA. 96-well ELISA plates were coated with 400 ng/well OVA antigen in 0.05 mol·L^{-1} carbonate buffer (pH 9.6) overnight at 4℃. These plates were washed three times with PBST (10 mmol·L^{-1} phosphate-buffered saline containing 0.1% Tween 20, pH 7.4) and blocked with 1.0 % of BSA in PBS for 60 min at 37℃. Aliquots of diluted serum samples (100 μl) were added to each well and incubated at 37℃ for 1 h followed by three washings with PBST. Sixty minutes after 100 μl of 1∶1000 dilution of HRP-GAM-IgG to was added each well, the plates were washed and 100 μl TMB substrate solution (10 ml TMB 0.2 g·L^{-1} and 50 μl 30% H_2O_2 in 10 ml citrate 0.1 mol·L^{-1}-phosphate buffer 0.2 mol·L^{-1}, pH 5.2) was added and reacted at room temperature in the dark for 10 min before 50μl of H_2SO_4 2 mol·L^{-1} was added. The colored reaction product was measured at 450 nm on a micro-ELISA Reader.

1.7 Statistical analysis

The data were expressed as $\bar{x} \pm s$. Statistical analysis of the difference between groups was evaluated by one way ANOVA of SPSS 13.0 statistical analysis software. *P* values less than 0.05 were considered statistically significant.

2 RESULTS

2.1 Carbohydrate contents and uronic acids contents of seven polysaccharides

The carbohydrate contents were determined by phenol-sulfuric acid method. The contents of carbohydrates and uronic acids in the seven polysaccharides were showed in Tab.1. The molecular mass of each polysaccharide was determined by HPLC-GPC method and showed in Fig.1. The results showed that these polysaccharides were markedly different in chemical compositions and molecular mass.

2.2 Effects of seven polysaccharides on production of OVA-specific serum antibody

After three-time immunization, serum samples collected from mice tails on d 21, d 52 and d 70 were used to detect antibody against OVA by ELISA.

Tab. 1 Contents of carbohydrates and uronic acids in seven polysaccharides

Polysaccharide	Carbohydrate contents/%	Uronic acids contents/%
IRPS	65.41	13.36
ARPS	30.88	19.73
APS	43.70	6.53
SPS	48.88	5.96
PPS	58.68	1.96
LCPS	45.83	8.96
EPS	32.60	7.53

APS: *Astragalus* polysaccharide; ARPS: *Angelica* root polysaccharides; EPS: *Epimedium* polysaccharides; IRPS: *Isatis* root polysaccharide; LCPS: *Lycium* polysaccharides; PPS: *Poria* polysaccharides; SPS: *Solomonseal* polysaccharides; GPC-gel permeation chromatography.

The OVA-specific antibody levels in sera were measured and shown in Fig. 2 – Fig. 4. The results suggested that after the first immunization the lower OVA-specific antibody titers were exhibited in all groups (Fig. 2). After the second immunization, the antibody titers of mice treated with EPS increased significantly ($P<0.01$) compared with those treated saline and alum, and the titers of PPS and IRPS groups also rose ($P<0.05$) (Fig. 3). However, other polysaccharides did not show adjuvant activities obviously. After the third immunization, the antibody titers in the serum of EPS groups were further enhanced, and the OVA-special antibody levels of IRPS, PPS and ARPS groups were also improved significantly, compared with OVA with saline, OVA with alum, and normal control groups ($P<0.01$) (Fig. 4). These data indicated that EPS, IRPS, PPS and ARPS could promote the production of antibody

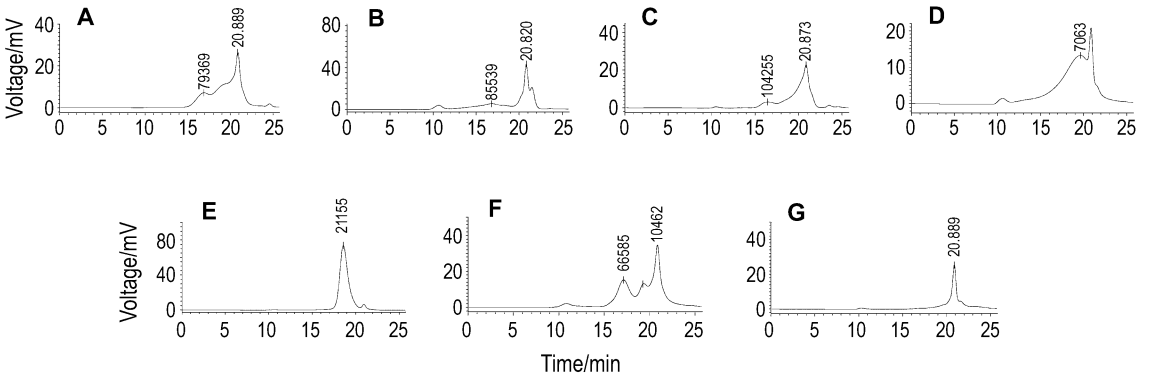

Fig. 1 Molecular mass character of IRPS(A), ARPS(B), APS(C), SPS(D), PPS(E), LCPS(F) and EPS(G) by HPLC with TSK-GELG4000 SWXL column. Mobile phase was Na_2SO_4 0.1 mol·L^{-1}; flow rate was 0.6 ml·min^{-1}; detection was refractive index detector; temperature was 30℃.

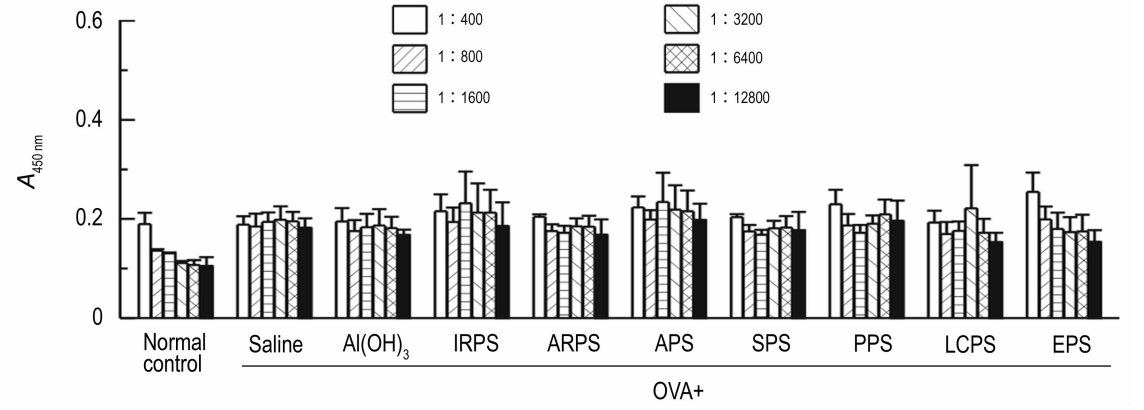

Fig. 2 OVA specific antibody titers in sera of immunized mice treated with seven polysaccharides after initial immunization. $\bar{x}\pm s$, $n=5$. $^{*}P<0.05$, $^{**}P<0.01$, compared with normal control group; $^{\#}P<0.05$, $^{\#\#}P<0.01$, compared with OVA + saline group; $^{\triangle}P<0.05$, $^{\triangle\triangle}P<0.01$, compared with OVA + alum group.

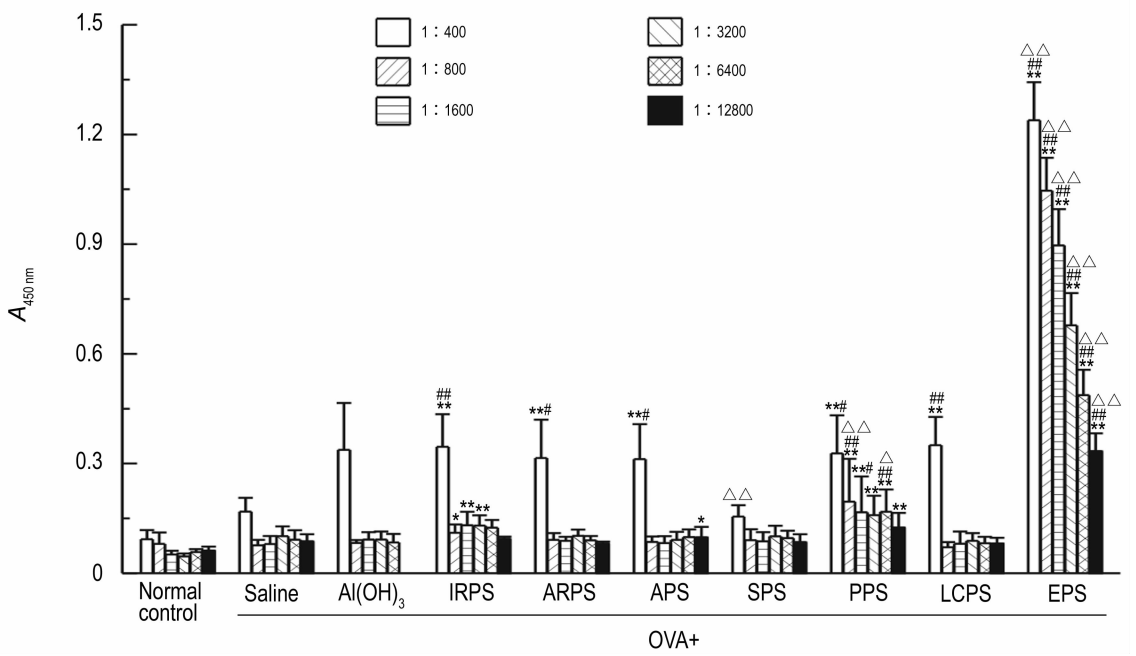

Fig. 3 OVA specific antibody titers in sera of immunized mice treated with seven polysaccharides after the second immunization. $\bar{x} \pm s$, $n=5$. $^{*}P<0.05$, $^{**}P<0.01$, compared with normal control group; $^{\#}P<0.05$, $^{\#\#}P<0.01$, compared with OVA + saline group; $^{\triangle}P<0.05$, $^{\triangle\triangle}P<0.01$, compared with OVA + alum group.

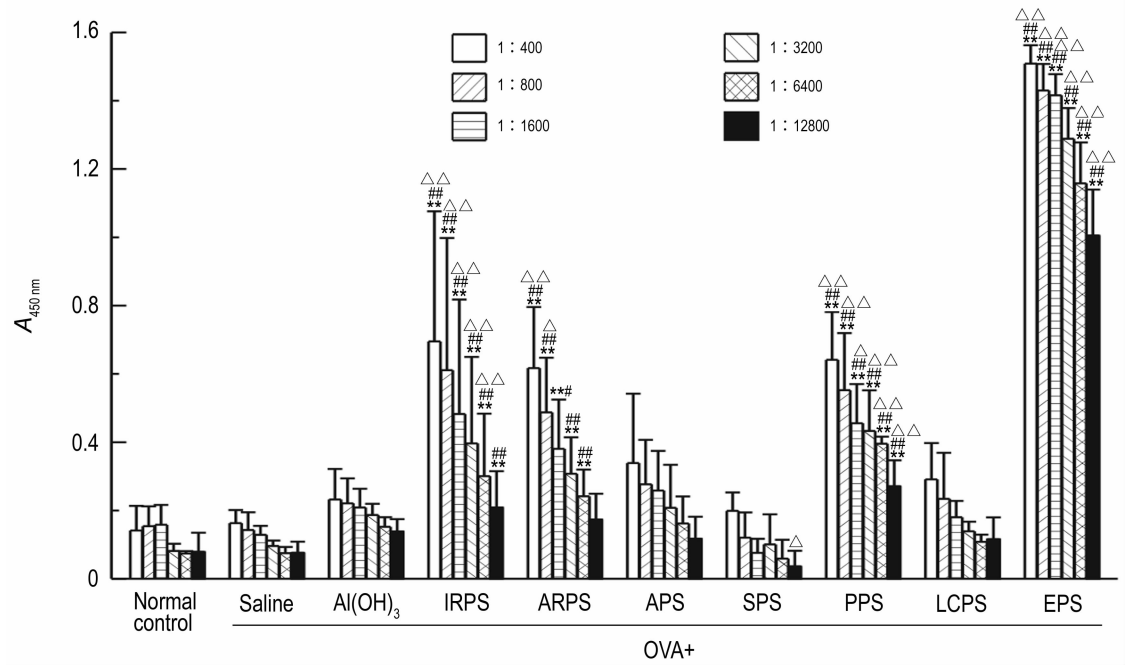

Fig. 4 OVA specific antibody titers in sera of immunized mice treated with seven polysaccharides after the third immunization. $\bar{x} \pm s$, $n=5$. $^{*}P<0.05$, $^{**}P<0.01$, compared with normal control group; $^{\#}P<0.05$, $^{\#\#}P<0.01$, compared with OVA + saline group; $^{\triangle}P<0.05$, $^{\triangle\triangle}P<0.01$, compared with OVA + alum group.

in the sera of mice immunized with OVA. In terms of the results of immune response, the strongest of the four polysaccharides was EPS, followed by IRPS, PPS and ARPS. However, APS, LCPS and SPS were not obvious in adjuvant effects.

3 DISCUSSION

Many of previous researches have found that some natural polysaccharides pronounce immunologic enhancement and can be used as immunopotentiators[18-19]. The representative polysaccharides were from astragalus, angelica and lycium[20-22]. Having been long used as folk medicines, these natural plants prove to be safe to humans and animals[23].

Some medical complexes containing plant polysaccharides have been successfully used as

the adjuvants to prepare vaccines. For example, Yang *et al*[24] researched two Chinese herbal medicinal ingredients (CHMI) for vaccine adjuvants against rabbit hemorrhagic disease. CHMI 1 contained an equal amount of epimedium polysaccharide (71.23% of glucose content) and propolis flavone while cCHMI2 had an equal amount of astragalus polysaccharides (88.96% of glucose content) and ginsenosides. The result showed that these ingredients could enhance serum antibody titers and lymphocyte proliferation. Their adjuvant effects were slightly superior to those of aluminum adjuvant. Fan *et al*[25] found that when chickens immunized with Newcastle disease vaccine were treated with a prescription containing epimedium polysaccharide (71.23% of glucose content) and propolis flavone, their serum antibody titers and lymphocyte proliferation were increased. Inoculated mice exhibited significantly decreased morbidity and mortality. Qiu *et al*[12] evaluated four Chinese herbal polysaccharides on the production of serum antibodies and the proliferation of peripheral T lymphocytes in vaccinated chickens. The result showed that low-dose astragalus polysaccharide and isatis root polysaccharide, and high-dose achyranthes root polysaccharide and Chinese yam polysaccharide could significantly enhance Newcastle disease antibody titers, proliferation activity of peripheral T lymphocytes, and the ratio of $CD4^+$ to $CD8^+$. Kong *et al*[26] also found that astragalus polysaccharide and isatis root polysaccharide could promote lymphocyte proliferation and antibody titers, but angelica polysaccharide exerted weaker effects on promoting immune responses.

The main component of poria polysaccharides is a β-(1→3)-D-glucan[27], Which was shown to induce proliferation of T lymphocytes and antibody production of B lymphocytes[28], increased the production of proinflammatory cytokines such as TNF-α, IL-6, and IL-1β in macrophages[29-30]. When the β-glucan was used as an adjuvant, mice and monkeys exerted higher levels of antibody against Venezuelan equine encephalomyelitis after the initial and second immunity[31-32]. Vanessa *et al*. connected β-glucan with bovine serum albumin (BSA) via a carbodiimide linkage and then immunized mice with this conjugate. A enhanced primary IgG antibody to BSA in mice was obtained[33].

Lycium barbarum, a well-known Chinese traditional medicine and foodstuff, has many proposed pharmacological and biological effects. Lycium polysaccharide is the main active component and possesses better immune enhancement[22]. Ling *et al*[34] purified a compound LBP3a from *Lycium b*, having a molecular mass of 8.2×10^4 ku. It was composed of α-D (1→4) polygalacturonan (92.8%), glucose (6.1%) and arabinose (1.1%). Mice immunized with DNA and LBP3a showed a significantly higher level of chlamydia clearance in the spleen and a greater Th1 immune response. Chen *et al*[11] isolated a polysaccharide-protein complex from *Lycium b* that induced phenotypic and functional maturation of DC with strong immunogenicity and up-regulated expression of CD40, CD80, CD86, and MHC class Ⅱ molecules.

In this study, we found that seven polysaccharides exerted very different molecular mass characters and significant difference in the contents of carbohydrate and uronic acids. These data suggested that the seven polysaccharides might initiate different degrees of immune response to OVA. It was evaluated the effect of seven polysaccharides on induction of humoral immune responses in OVA immunized mice for three times. After the initial immunity, the seven polysaccharides and alum could not elicit significant adjuvant responses for OVA. After in the second immunization, epimedium polysaccharide induced high levels of OVA-specific antibody, and poria polysaccharides and isatis polysaccharides stimulated a slight antibody response. Fourteen days after the third immunization, the OVA-specific antibody titer of mice in epimedium polysaccharides group was further increased, and the anti-OVA antibody levels in the serum of mice treated with isatis polysaccharides, angelica polysaccharides and poria polysaccharides improved significantly compared with OVA with alum and OVA with saline controls. During the period of three time′s immunity, no significant toxicity was observed in the mice treated with OVA plus seven polysaccharides.

In summary, the seven isolated polysaccharides from seven Chinese herbs displayed adjuvant functions, and four polysaccharides, especially epimedium polysaccharides, could promote humoral immune response. As promising adjuvant candidates, these polysaccharides should be further investigated.

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7 种植物多糖对小鼠卵清蛋白免疫反应的佐剂活性

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摘要: 目的 寻找和筛选具有较好佐剂功能的天然多糖。**方法** 自中药提取当归多糖、黄芪多糖、板蓝根多糖、茯苓多糖、黄精多糖、枸杞多糖和淫羊藿多糖。苯酚硫酸法测总糖含量;间羟联苯法分析糖醛酸含量;凝胶渗透色谱法测定多糖分子量分布。按照分组,每只 BALB/c 小鼠肌内注射 60 μg 卵清蛋白(OVA), OVA +0.1 mg 氢氧化铝,OVA +1 mg多糖,OVA + 生理盐水,并分别在第 1, 28 和 58 天进行 3 次注射,在第21, 52, 70 天采血,测定血清 OVA 抗体滴度。**结果** 7 种多糖中的总糖含量为板蓝根总糖,65.41%; 当归总糖, 30.88%;黄芪总糖, 43.70%;黄精总糖,48.88%;茯苓总糖, 58.68%;枸杞总糖, 45.83%; 淫羊藿总糖, 32.60%;总糖中糖醛酸含量为板蓝根糖醛酸,13.36%; 当归糖醛酸, 19.73%;黄芪糖醛酸, 6.53%;黄精糖醛酸, 5.96%;茯苓糖醛酸, 1.96%;枸杞糖醛酸, 8.96%;淫羊藿糖醛酸, 7.53%。7 种多糖表现出不同的分子质量分布特征。初次免疫,7 种多糖和铝佐剂均未激活小鼠血清 OVA 抗体产生;在第 2 次免疫后,淫羊藿多糖佐剂组产生较高滴度的抗体,达到 $1:10^5$;第 3 次免疫后淫羊藿多糖佐剂组抗体滴度进一步提高,板蓝根多糖、当归多糖及茯苓多糖佐剂组的 OVA 特异性抗体滴度均达到 $1:10^5$,与 OVA +0.1 mg氢氧化铝及 OVA + 生理盐水组比较有显著差异($P<0.05$)。**结论** 板蓝根多糖、当归多糖、茯苓多糖及淫羊藿多糖均具有很好的佐剂作用,特别是淫羊藿多糖具有较强的激发体液免疫活性。多糖有望成为候选的新型免疫佐剂。

关键词: 多糖, 当归; 多糖, 黄芪; 多糖, 板蓝根; 多糖, 茯苓; 多糖, 黄精; 多糖, 枸杞; 多糖, 淫羊藿; 免疫; 佐剂

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