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Effects of Astragalus polysaccharide liposome on lymphocyte proliferation in vitro and adjuvanticity in vivo

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ABSTRACT

Two experiments were carried out. In vitro, the effects of Astragalus polysaccharide liposome (APSL) on chicken's T and B lymphocytes proliferation were determined. The results showed that APSL could significantly promote T and B lymphocytes proliferation singly or synergistically with PHA and LPS and the efficacy were superior to those of Astragalus polysaccharide (APS) and blank liposome (BL) at most concentrations. In immune response experiment, the adjuvanticity of APSL at three doses, APS and BL were compared on chickens vaccinated with ND vaccine. The results showed that APSL could significantly promote lymphocyte proliferation, enhance antibody titer, promote IFN-γ, IL-2, IL-4 and IL-10 secretion, and its medium dose possessed the best efficacy. These results indicated that APSL could significantly improve the adjuvanticity and drug action of APS, and its medium dose possessed the best efficacy, the liposome would be expected to exploit into a new-type preparation of APS.

1. Introduction

Radix Astragali is the dried roots of Astragalus membranaceus (Fisch) Bge. Var. mongholicus (Bge) Hsiao or Astragalus membranaceus (Fisch) Bge. The usage of Radix Astragali could be dated back more than 2000 years ago and recorded in Shen Nong's Materia Medica written in the Han dynasty. The Radix Astragali is one of the most popular health-promoting herbal medicines commonly used in China. Scientific investigation in the last two decades has revealed much insight into the pharmacological functions of different components of Radix Astragali, especially its polysaccharide fractions (Bedir, Pugh, Calis, Pasco, & Khan, 2000; Shimizu, Tomoda, Kanari, & Gonda, 1991). Astragalus polysaccharide (APS) possesses immune enhancement both in vivo and in vitro, such as promoting T and B lymphocytes proliferation induced by concanavalin A (ConA) and lipopolysaccharide (LPS) (Gao, Cao, Li, Tong, & Sa, 2001); enhancing the immune organ index, the transformation of T lymphocytes (Chen, Zhuo, Li, Chang, & Zhang, 2003), the activities of B lymphocyte and NK cell (Shao, Zhao, Chen, & Pang, 2006), and the antibody titer (Kong, Hu, Rui, Wang, & Li, 2004); promoting the cytokine expression of IL-2, IL-4, and IFN-γ (Qiu, Cui, Hu, Dong, & Zhang, 2009); improving the immune defensive function and so on. At present, APS as immunologic adjuvant has been used widely in clinical application of veterinary diseases.

Nowadays, most part of the APS preparations has been metabolized or eliminated in the distribution process before they arrive at the site of action, and the effective blood drug level could only occupy a short time during using drugs. Therefore, it needs to be used with large dose or successively (Li, Yang, & Li, 2008). But successive administration has many disadvantages, for example, it not only consumes the manpower, but also causes stress of animal. The curve of blood drug level presents obvious changes from wave ridge to trough, which increases the risk of adverse effect on animal. The drug action is reduced because the schedule time and defined dosage are not kept strictly in clinical use which leads to the emergence of drug resistant strain and finally shortens the service life of new veterinary drugs (Wu, Jin, Zhao, Gao, & Liao, 2009). Therefore, it is great significance to research new-type preparations or select a highly effective carrier of APS in its clinical application.

The liposome has been studied widely in recent years as the carrier of drugs for its advantaged superiority, such as targeting and slow releasing, no immunogenicity, low toxicity, improved bioavailability and so on (Wang, Qiu, & Xu, 2004). After hypodermic or intramuscular injection, the liposome can be detained or ingested by lymph vessel and blood capillary at the injection site,

Abbreviations: APS, Astragalus polysaccharide; APSL, Astragalus polysaccharide liposome; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate buffered saline; PHA, phytohemagglutinin; LPS, lipopolysaccharide; DMSO, dimethyl sulfoxide; ND, Newcastle disease; BC, blank control; BL, blank liposome; VC, vaccine control; IFN-γ, interferon-γ; IL-2, interleukin-2; IL-4, interleukin-4; IL-10, interleukin-10.

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instead of being absorbed into bloodstream directly. Moreover, the liposome can also release drugs after hydrolyzed at the injection site and maintain the drug concentration in a high level for a long time (Wu et al., 2009). Based on the above characteristic of liposome, it is introduced into APS adjuvant. The purpose is to combine the physiological function of liposome with the immune enhancement of APS to improve the adjuvanticity and drug action of APS.

In this study Astragalus polysaccharide liposome (APSL) and APS were prepared. In vitro, the effects of APSL on chicken's T and B lymphocytes proliferation were determined by MTT method, taking APS as control. In immune response test, the chickens were injected with APSL simultaneously with inoculating Newcastle disease (ND) vaccine, taking APS and blank liposome (BL) as controls. The changes of the lymphocytes proliferation, antibody titers and concentrations of four kinds of cytokines in serum were measured. The objective of this research was to investigate the possibility for APSL to promote the adjuvanticity of APS and determine the best dosage for clinical use.

2. Materials and methods

2.1. Astragalus polysaccharide

Astragalus polysaccharide (APS, net content of 87.7%) was prepared in our laboratory. In vitro, it was diluted into five working concentrations (125–7.813 $\mu g\,mL^{-1}$) in two-fold serial dilution with RPMI-1640 containing 10% fetal bovine serum, sterilized and stored at 4 °C. In vivo, APS was diluted into 4 $mg\,mL^{-1}$ with deionized water (Key laboratory of Nanjing Agricultural University), and the endotoxin amount was up to the standard of Chinese Veterinary Pharmacopoeia (less than 0.5 $EU\,mL^{-1}$) (Veterinary Pharmacopoeia commission of the People's Republic of China, 2011).

2.2. Preparation of APSL

The mixture of APS, soybean phospholipid, cholesterol and tocopherol was dissolved in ethanol–chloroform solution (1:1, v/v), and poured into round bottom flask. The solution was evaporated to dryness in 40 °C water bath by rotary evaporator (Model RE-52A, Yarong Biochemical Instrument Manufacturer, Shanghai City), and finally a dry film was formed in the sidewall. The APS dissolved in phosphate buffered saline (PBS, pH 7.4) was poured into the round bottom flask, which was joggled until the film was completely dissolved at room temperature (25 °C). Ultimately, the crude suspension obtained, then was passed through 0.8 μm, 0.45 μm and 0.22 μm millipore membrane filters successively (Gunter, Gunter, Jarkowski, & Rosier, 1982; Zhang, Anyarambhatla, & Ma, 2005). The final liposome suspension was transferred into vials and lyophilized by vacuum freeze-drying machine (Model LGJ-25, Dongxing Machinery Industry Co., Ltd. Shamen City).

APSL powder showed porous massive structure. After the hydration, it became the uniform translucent suspension. Under the transmission electron microscope, particles presented spherical or nearly spherical shape with uniform size whose diameters were less than 200 nm. The entrapment rate of APS was 49.93%. In vitro, APSL was diluted into five working concentrations (125–7.813 $\mu g\,mL^{-1}$) in two-fold serial dilution with RPMI-1640 containing 10% fetal bovine serum. In vivo, it was divided into high (4.0 $mg\,mL^{-1}$), medium (2.0 $mg\,mL^{-1}$) and low (1.0 $mg\,mL^{-1}$) dose with physiological saline.

2.3. Reagents and vaccine

Soybean phospholipid (No. 20090728) was manufactured by Shanghai Taiwei Pharmaceutical Co., Ltd. Cholesterol (No. 20090908) was purchased from Anhui Tianqi Chemical Technology Co., Ltd. Protamine (Sigma, P4380) was dissolved by physiological saline to 10 $mg\,mL^{-1}$. Lymphocyte separation medium (No. 090818) was manufactured by Shanghai Huajing Biology Inc. RPMI-1640 (GIBCO) with the supplement of 100 IU mL^{-1} benzylpenicillin, 100 IU mL^{-1} streptomycin and 10% fetal bovine serum was used for washing and re-suspending cells, diluting mitogen and culturing the cells. Phytohemagglutinin (PHA, Sigma, No. L8754), as a T-cell mitogen, was dissolved into 0.1 $mg\,mL^{-1}$ with RPMI-1640. Lipopolysaccharide (LPS, Sigma, No. L2880), as a B-cell mitogen, was dissolved into 0.05 $mg\,mL^{-1}$ with RPMI-1640. Hanks' solution was used for diluting blood. The 3-(4,5-dimethylthiazo l-2-yl)-2,5-diphenyltetrazolium bromide (MTT, American Co.) was dissolved into 5 $mg\,mL^{-1}$ with calcium and magnesium-free (CMF) phosphate-buffered saline (PBS, pH 7.4). These reagents were filtered through a 0.22 μm millipore membrane filter. PHA and LPS solutions were stored at −20 °C, MTT solution, at 4 °C in dark bottles, RPMI-1640 were stored at 4 °C. Dimethyl sulfoxide (DMSO, No. 090601) was produced by Kemiou Institute of Chemical Engineering in Tianjin. Other chemicals used in experiments were of analytical grade.

ND vaccine (La Sota strain, No. 091118) was purchased from Institute of Veterinary Medicine, Jiangsu Academy of Agricultural Science.

2.4. The design of experiment in vitro

2.4.1. T lymphocyte proliferation assay

Blood samples were collected from non-immunized White Roman chickens (male) at 60-day-old (provided by Tangquan Poultry Farm) and transferred immediately into aseptic capped tubes containing sodium heparin, then diluted with an equal volume of Hanks' solution and carefully layered on the surface of lymphocyte separation medium. After centrifugation at 800 × *g* for 20 min, the lymphocytes was collected and washed twice with RPMI-1640 without fetal bovine serum. The resulting pellet was re-suspended and diluted to 5×10^6 mL^{-1} with RPMI-1640 after the cell viability was assessed by trypan blue exclusion. The solution was divided into two parts, one part was added with PHA, and respectively incubated into 96-well culture plates, 100 μL per well. Then, APSL, APS and BL at series of concentrations were added, in cell control group and PHA control group, RPMI-1640 and PHA respectively, 100 μL per well, four wells each concentration. The final concentration of PHA reached to 20 $\mu g\,mL^{-1}$. The plates were incubated in a humid atmosphere with 5% CO2 (Revco, Co., USA) at 39.5 °C for 48 h. Briefly, 20 μL of MTT (5 $mg\,mL^{-1}$) was added into each well at 4 h before the end of incubation. Then the plates were centrifuged at 1000 × *g* for 10 min at room temperature. The supernatant was removed carefully and 100 μL of DMSO was added into each well. The plates were shaken for 5 min to dissolve the crystals completely. The absorbance of cells in each well was measured by microliter enzyme-linked immunosorbent assay reader (Model RT-6000, Leidu Co., Ltd. Shenzhen City) at a wave length of 570 nm (A_{570} value) as the index of lymphocytes proliferation (Wang, Hu, Kong, et al., 2005; Wang, Hu, Sun, et al., 2005).

2.4.2. B lymphocyte proliferation assay

Non-immune White Roman chicken (male) at 60-day-old was put to death, the spleen was taken out aseptically and washed twice with physiological saline, then immediately transferred into a Petri dish with Hanks' solution, and cut into 1–2 mm^3 pieces, the tissues were blown fully with pipette which made the cell suspended in Hanks' solution, then filtered through 3-tier gauze. Finally, the Hanks' solution contained cell was collected and carefully layered on the surface of lymphocyte separation medium. Then the treatment and determinations were similar to 2.4.1. (T lymphocyte proliferation assay) except that PHA (final concentration,

20 μg mL^{-1}) was instead of LPS (final concentration, 10 μg mL^{-1}) (Zhao et al., 1995).

2.5. The design of experiment in vivo

2.5.1. Animals

One-day-old White Roman chickens (male) purchased from Tangquan Poultry Farm were housed in wire cages (60 cm × 100 cm) in air-conditioned rooms at 37 °C and lighted for 24 h at the beginning of pretrial period. The temperature was gradually declined to the room temperature and the light time to 12 h per day, which were kept constantly in the following days. Chickens were fed with the commercial starter diet provided by the feed factory of Jiangsu Academy of Agricultural Science.

2.5.2. Immune response test

Three hundred and fifty 14-day-old chickens were randomly divided into 7 groups and vaccinated with Newcastle disease vaccine except for blank control (BC) group, repeated vaccination at the age of 28 days. At the same time of the first vaccination, the chickens in five experimental groups were intramuscularly injected respectively with 0.5 mL of APSL at high (4.0 mg mL^{-1}), medium (2.0 mg mL^{-1}) and low (1.0 mg mL^{-1}) dose, APS at 4.0 mg mL^{-1} and BL, in vaccine control (VC) and BC groups, 0.5 mL of physiological saline. On days 7, 14, 21 and 28 after the first vaccination, the blood was sampled for determination of lymphocyte proliferation (same to 2.4.1.) and serum hemagglutination inhibition (HI) antibody titer. On days 14, 21 and 28 after the first vaccination, 6 chickens were sampled randomly from each group for determining the concentrations of interferon-γ (IFN-γ), interleukin-2 (IL-2), interleukin-4 (IL-4) and interleukin-10 (IL-10) in serum by Enzyme-linked Immunosorbent Assay (ELISA).

2.6. Serum HI antibody assay

Blood samples (1.0 mL per chick) were collected from the brachial vein, put into 1.5 mL of Eppendorf tubes and allowed to clot at 37 °C for 2 h. The serum was separated and stored at −20 °C for HI antibody assay. Briefly, two-fold serial dilution of 50 μL serum, after inactivated at 56 °C for 30 min, were made in a 96-well, V-shaped bottom microtiter plate containing 50 μL of CMF-PBS in each well, then 50 μL of NDV antigen (4 haemagglutination, HA units) was added into all wells except for the last row as controls. Serum dilutions ranged from 1:2 to 1:2048. The antigen-serum mixture was incubated for 10 min at 37 °C, then 50 μL of 1% rooster erythrocytes suspension was added into each well and continued to incubate for 30 min. A positive serum, a negative serum, erythrocytes and antigens were also included as controls. The highest dilution of serum causing complete inhibition was considered the endpoint. The geometric mean titer was expressed as reciprocal $\log_2$ values of the highest dilution that displayed HI (Guo et al., 2009).

2.7. Serum cytokines concentration assay

Blood samples (2.0 mL per chick) from brachial vein were drawn into Eppendorf tube and allowed to clot at 37 °C for 2 h. Serum was separated by centrifugation for determination of the concentrations of cytokines. The concentrations of IFN-γ, IL-2, IL-4 and IL-10 in serum were assayed by ELISA Kit (ADL, Co., USA).

2.8. Statistical analysis

Data are expressed as the mean ± S.D. Duncan's multiple range test was used to determine the differences among groups with the software SPSS 16.0. Significant differences were considered at $P < 0.05$.

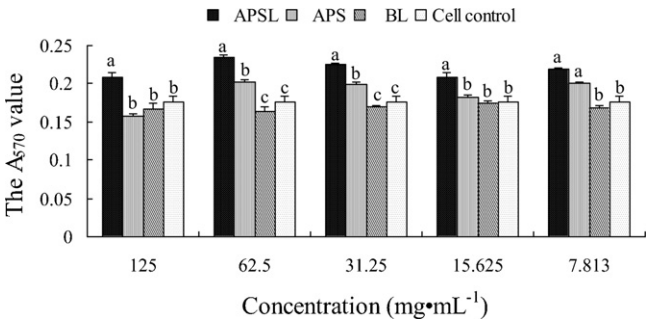

Fig. 1. Changes of T lymphocyte proliferation in single stimulation of drugs (A_{570} value). [a–c]Bars in the same concentration without the same superscripts differ significantly ($P < 0.05$).

3. Results

3.1. The test in vitro

3.1.1. Changes of T lymphocyte proliferation in single stimulation of drugs

The results are illustrated in Fig. 1. At 125–15.625 μg mL^{-1}, the A_{570} values of APSL group were significantly higher than those of APS group ($P < 0.05$), and at 125–7.813 μg mL^{-1}, significantly higher than those of BL and cell control groups ($P < 0.05$). The A_{570} values of APS group were significantly higher than those of BL and cell control groups at 62.5, 31.25 and 7.813 μg mL^{-1} ($P < 0.05$).

3.1.2. Changes of T lymphocyte proliferation in synergistical stimulation of drugs with PHA

The data are shown in Fig. 2. At 125–31.25 μg mL^{-1}, the A_{570} values of APSL group were significantly higher than those of APS group ($P < 0.05$) and at 125–15.625 μg mL^{-1}, significantly higher than those of BL and cell control groups ($P < 0.05$). The A_{570} values of APS group were significantly higher than those of PHA control group at 62.5 and 31.25 μg mL^{-1}. At 62.5 μg mL^{-1}, the A_{570} value of APS group was significantly higher than that of BL group ($P < 0.05$).

3.1.3. Changes of B lymphocyte proliferation in single stimulation of drugs

The results are illustrated in Fig. 3. At 62.5 and 31.25 μg mL^{-1}, the A_{570} values of APSL group were significantly higher than those of APS group, significantly higher than those of BL group at 62.5–7.813 μg mL^{-1} ($P < 0.05$) and those of cell control group at 125–7.813 μg mL^{-1} ($P < 0.05$). The A_{570} values of APS group were significantly higher than those of cell control group at 31.25–7.813 μg mL^{-1} ($P < 0.05$).

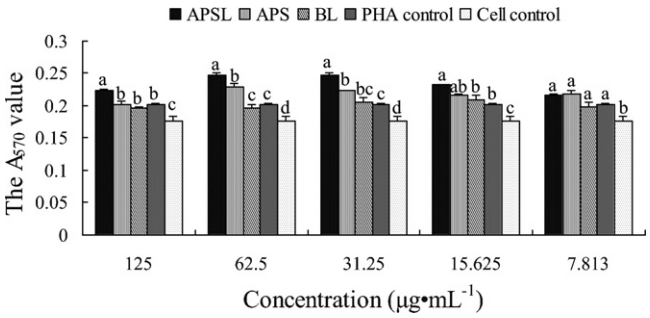

Fig. 2. Changes of T lymphocyte proliferation in synergistical stimulation of drugs with PHA (A_{570} value). [a–d]Bars in the same concentration without the same superscripts differ significantly ($P < 0.05$).

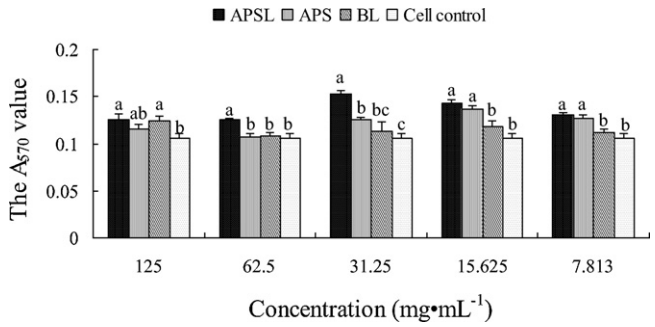

Fig. 3. Changes of B lymphocyte proliferation in single stimulation of drugs (A_{570} value). $^{a-c}$Bars in the same concentration without the same superscripts differ significantly ($P < 0.05$).

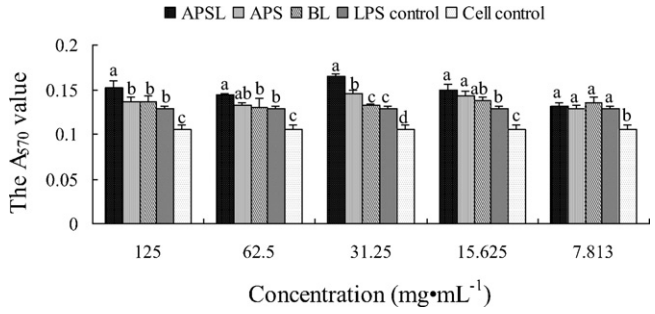

Fig. 4. Changes of B lymphocyte proliferation in synergistical stimulation of drugs with LPS (A_{570} value). $^{a-d}$Bars in the same concentration without the same superscripts differ significantly ($P < 0.05$).

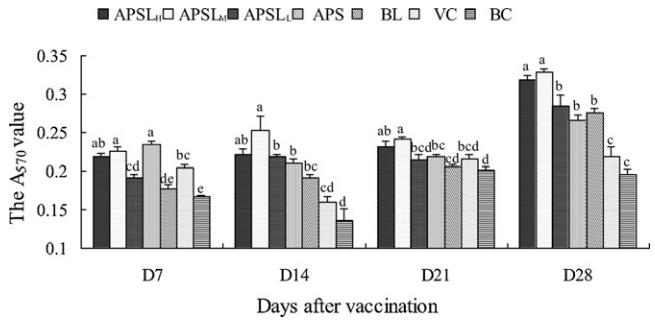

Fig. 5. The changes of lymphocyte proliferation in each group (A_{570} value). $^{a-e}$Bars in the same day without the same superscripts differ significantly ($P < 0.05$).

3.1.4. Changes of B lymphocyte proliferation in synergistical stimulation of drugs with LPS

The changes of A_{570} values are showed in Fig. 4. At 125 and 31.25 μg mL^{-1}, the A_{570} values of APSL group were significantly higher than those of APS group and significantly higher than those of BL, LPS and cell control groups at 125–31.25 μg mL^{-1} ($P < 0.05$). The A_{570} values of APS group were significantly higher than those of LPS control group at 31.25 and 15.625 μg mL^{-1} ($P < 0.05$).

3.2. The test in vivo

3.2.1. The changes of lymphocyte proliferation

The A_{570} values in each group are shown in Fig. 5. On days 14–28, the A_{570} values in APSL$_M$ groups were the highest and significantly higher than those in APS, APSL$_L$, BL, VC and BC groups ($P < 0.05$). On day 7, the A_{570} value in APS group was significantly higher than those in APSL$_L$, BL, VC and BC groups. On days 14 and 28, the A_{570} values in APS group were only significantly higher than those in VC and BC groups ($P < 0.05$).

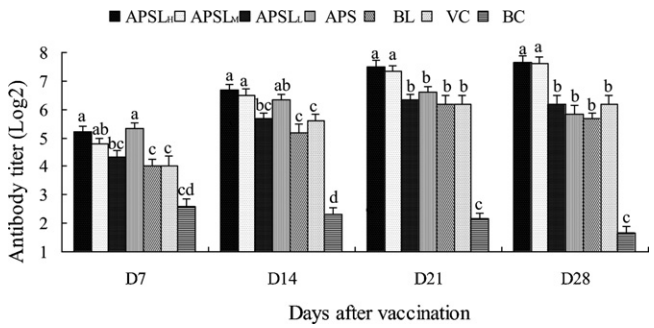

Fig. 6. The changes of antibody titer in each group (Log_2). $^{a-d}$Bars in the same day without the same superscripts differ significantly ($P < 0.05$).

3.2.2. The changes of antibody titer

The changes of antibody titers are shown in Fig. 6. On days 21 and 28, the antibody titers in APSL$_H$ and APSL$_M$ groups were significantly higher than those in APS, APSL$_L$, BL, VC and BC groups ($P < 0.05$). On day 14, the antibody titers in APSL$_H$ and APSL$_M$ groups were higher than that in APS group. On days 7 and 14, the titers in APS group were significantly higher than those in BL, VC and BC groups ($P < 0.05$).

3.2.3. The changes of IFN-γ concentrations

The serum IFN-γ concentrations in each group are illustrated in Fig. 7(a). On days 21 and 28 after the first vaccination, the IFN-γ concentrations in APSL$_M$ group were significantly higher than those in APS, APSL$_L$, BL, VC and BC groups ($P < 0.05$). On days 14–28, the IFN-γ concentrations in APSL$_H$ and APSL$_M$ groups were significantly higher than those in APSL$_L$ group ($P < 0.05$). The IFN-γ concentration in APSL$_H$ group was significantly higher than that in APS group only on day 28 ($P < 0.05$). On days 14 and 21, the IFN-γ concentrations in APS group were significantly higher than those in VC and BC groups ($P < 0.05$).

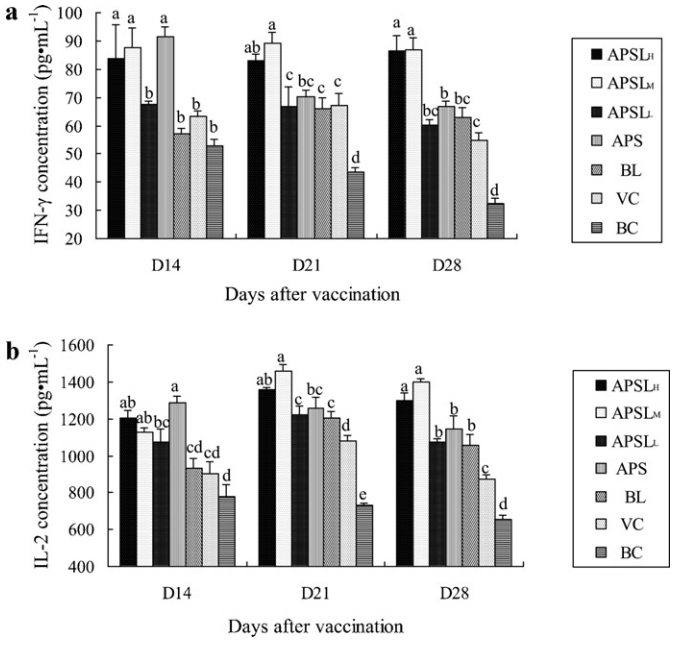

Fig. 7. (a). The changes of IFN-γ concentration in each group (pg mL^{-1}). (b). The changes of IL-2 concentration in each group (pg mL^{-1}). $^{a-d}$Bars in the same day without the same superscripts differ significantly ($P < 0.05$).

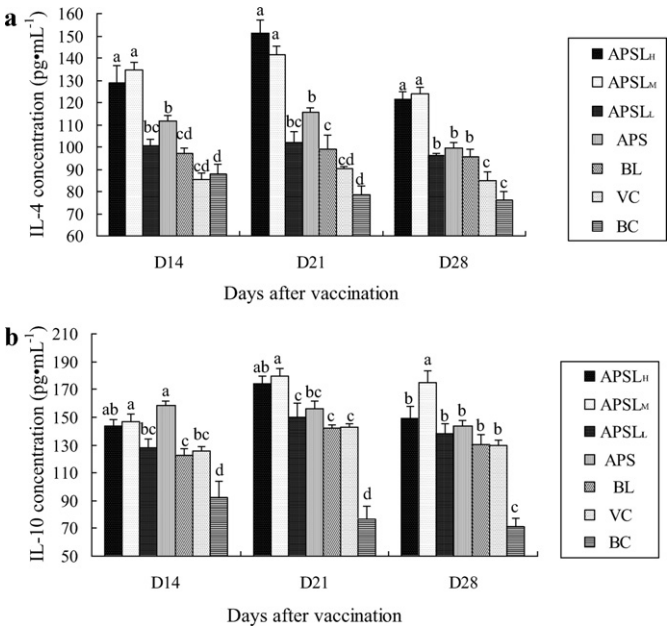

Fig. 8. (a). The changes of IL-4 concentration in each group (pg mL^{-1}). (b). The changes of IL-10 concentration in each group (pg mL^{-1}). $^{a-d}$Bars in the same day without the same superscripts differ significantly ($P < 0.05$).

3.2.4. The changes of IL-2 concentrations

The results are illustrated in Fig. 7(b). On days 21 and 28 after the first vaccination, the IL-2 concentrations in $APSL_M$ group were significantly higher than those in APS, $APSL_L$, BL, VC and BC groups ($P < 0.05$), in $APSL_H$ and $APSL_M$ groups were significantly higher than those in $APSL_L$ group. The IL-2 concentration in $APSL_H$ group was significantly higher than that in APS group only on day 28 ($P < 0.05$). On days 14–28, the IL-2 concentrations in APS group were significantly higher than those in VC and BC groups ($P < 0.05$).

3.2.5. The changes of IL-4 concentrations

The changes of IL-4 concentrations are shown in Fig. 8(a). On days 14–28 after the first vaccination, the IL-4 concentrations in $APSL_H$ and $APSL_M$ groups were significantly higher than those in APS, $APSL_L$, BL, VC and BC groups ($P < 0.05$), in APS group were significantly higher than those in VC and BC groups.

3.2.6. The changes of IL-10 concentrations

The changes of IL-10 concentrations are shown in Fig. 8(b). On day 21 after the first vaccination, the IL-10 concentration in $APSL_M$ group was significantly higher than those in APS, $APSL_L$, BL, VC and BC groups ($P < 0.05$). On day 28, the IL-10 concentration in $APSL_M$ group was significantly higher than those in APS, $APSL_H$, $APSL_L$, BL, BC and BC groups ($P < 0.05$). On day 14, the IL-10 concentration in APS group was significantly higher than those in $APSL_L$, BL, VC and BC groups ($P < 0.05$).

4. Discussion

Lymphocytes proliferation is the most direct indicator of reflecting the state of cellular immunity. T and B lymphocytes play an important role in enhancing immune function of organism (Minato, Kawakami, Nomura, Tsuchida, & Mizuno, 2004). Therefore, the determination of the changes of T and B lymphocytes proliferation is the better method to study the activity and action mechanism of drugs. Because the experiment in vitro takes relatively short time and its conditions are easily controlled (He, 2002), it is suitable for initial screening of drugs with immune enhancement. The results of this experiment in vitro showed that APSL group presented the highest A_{570} values at most concentrations whether stimulations were single or synergistical with PHA and LPS, and were significantly higher than those of APS group, which indicated that APSL possessed the strongest effect, and the immune activity of APS was significantly improved after being encapsulated by liposome. Cai, Peng, Chen, and Pang (2009) reported that lentinan liposome could promote the transformation of T lymphocytes, and its effect was significantly better than that of lentinan injection, Li (2008) also proved that the polysaccharide of cistanche deserticola liposome could significantly promote T lymphocytes proliferation when polysaccharide of cistanche deserticola was encapsulated by liposome. These experimental results indicated that the efficacy of liposome carried drug was significantly better than that of free drug, and which was consistent with our experimental results.

The effect of Chinese herbal medicine on enhancing immune function is probably due to its direct action on immune system or indirect effect on other systems (He, 2002). In order to validate whether the effects in vitro and in vivo were consistent or not, the experiment in vivo must be conducted. The results of immune response test revealed that the A_{570} values in medium dose of APSL group were the highest and significantly higher than those in APS, BL, VC and BC groups at most time points. This indicated that APSL at suitable dose had the best efficacy in promoting lymphocytes proliferation to enhance cellular immunity and improving the adjuvanticity of APS. Therefore, the experimental results proved that the effects of APSL in vitro and in vivo were consistent from the aspect of cellular immunity.

Humoral immunity mediated by B lymphocyte is one of the main factors to resist infectious diseases as an important specific immune response (Qiu, Hu, Cui, Zhang, & Wang, 2007). The antibody level in poultry is the marker reflected humoral immune function. The results of the immune response test showed that on days 21 and 28 after the first vaccination, the antibody titers in high and medium doses of APSL group were significantly higher than those in other five groups, which confirmed that APSL possessed the best efficacy on enhancing humoral immunity at the suitable dose, and then improved the immune effect of ND vaccine and the adjuvanticity of APS. The results of the test in vitro also confirmed that APSL could promote B lymphocyte proliferation, and the effect was better than APS. Therefore, the experimental results proved that the effects of APSL in vitro and in vivo were consistent from the aspect of humoral immunity.

In order to further investigate the adjuvanticity of APSL, four kinds of cytokines relating to the immunoregulatory activity were selected, and the effect of APSL on secretion of them was studied. IFN-γ and IL-2 which are mainly secreted by the activated T lymphocyte possess great immunoregulatory effect and can promote the proliferation and differentiation of T cell (Yang, 2003). They belong to the Th1 cells and mediate cellular immunity. So the changes of IFN-γ and IL-2 concentrations in serum can be regarded as the important symbol for cellular immunity function (Xu & Li, 2007; Yang, Qin, & Zhu, 2009). IL-4 and IL-10 which belong to the Th2 cells mainly play an important role in humoral immunity, as they can promote the proliferation of B cell and the production of antibody (Salgame et al., 1991; Yang, 2003). As important immune correlation factors of Th1 and Th2 cells, IFN-γ, IL-2, IL-4 and IL-10 can reflect the basic status of cellular immunity and humoral immunity of organism to certain extent. The experimental results showed that the concentrations of the four cytokines in medium dose of APSL group were significantly higher than those in APS group at most time points. It indicated that the ability of APSL on promoting cytokines secretion was stronger than that of APS at proper doses. It was reported that the dynamic balance and mutual adjustment between Th1 and Th2 played an important role in maintaining normal immunologic function of organism (Qiu, Cui, Hu, Dong, & Zhang, 2009; Ying, Kikuchi, Meng, Kay, & Kaplan, 2002).

The experimental results also revealed that IFN-γ and IL-2 concentrations were increasing along with the increasing of IL-4 and IL-10 concentrations, which kept the dynamic balance of the Th1 cell and the Th2 cell to some extent.

Some scholars reported that liposome could alter the uptake of drug on cell in vivo and vitro, and the drugs encapsulated by liposome were able to be transported into the cell in several ways which might involve different mechanisms (Garelli, Vierling, Fischel, & Milano, 1993; Weistein & Leserman, 1984). Although APS could enhance the immune function of organism and had better efficacy, the experimental results confirmed that the immune enhancement effect of APSL was obviously better than APS. The reasons may be that on one hand, as the principal constituents of liposome, the phosphatide and cholesterol are also the main ingredients of the cell membrane, thus help the interactions between liposome and cell, such as absorption, fusion, lipid exchange, endocytosis and so on (Wang, Zhu, & Liao, 2007). Therefore, the APS encapsulated by liposome is easy to be absorbed by cell. On the other hand, the particle size of APSL is small and quite homogeneous, and the average particle diameter is less than 200 nm, so it has the characteristic of the nanophase material, which is more advantageous in penetrating cell membrane and being absorbed by cell. When the liposome preparation enters into the organism, it can decelerate the drug release and lengthen the action time of drug in organism by preventing the drug from degrading rapidly. Many other researches in vivo also confirmed that the efficacy of drug encapsulated by liposome was significantly better than free drug (Deng, 1996; Liu & Guo, 1999; Lee, Chung, & Lee, 2008), which was consistent with this experimental result.

This study also found that the adjuvanticity of APSL presented a definite dose–efficacy relationship. In promoting lymphocytes proliferation and enhancing the antibody titer, the high and medium doses of APSL at most time points were significantly better than its low dose. In inducing the secretion of IFN-γ, IL-2, IL-4 and IL-10, the high and medium doses of APSL at most time points were also superior to its low dose. The adjuvanticity of APSL at medium dose was significantly better than APS at most time points, but the effect of its high dose was similar to APS. Therefore, APSL at medium dose possessed the best efficacy. The dose-effect and time-effect relationships of drugs were also confirmed in our previous researches (Kong et al., 2004).

In conclusion, APSL could significantly improve the immune efficacy of ND vaccine and adjuvanticity of APS. Its medium dose had the best efficacy. Therefore, the liposome can be used as a carrier of APS and APSL would be expected to exploit into a new-type preparation of APS.

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