Immunomodulating effects of polysaccharides isolated from herbal products

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Abstract

Polysaccharides (PSs) have been isolated from herbs *Angelicae Radix* and *Hoelen,* and their immunomodulating effects studied. Angelica PS consisted mainly of glucose, galactose, rhamnose arabinose and galacturonic acid whereas Hoelen PS contained galactose, glucose, mannose and galacturonic acid.

The mice injected with Angelica or Hoelen PS, 0.2-1.0 mg/dose, i.p. followed by administration of pneumococcal type 9V PS-protein conjugate, 5 μg/dose, i.p. produced higher serum levels of 9V PS IgG and IgM Abs than the non-treated control group. The mice treated with plant PS showed more rapid bacterial clearance from blood after challenge with virulent type 19F pneumococci. In addition, in mice immunized with type 9V glycoconjugate the treatment of plant PS induced TNF-α 4.0 to 5.5 fold higher than the control group. In immunized mice treated with Hoelen PS, 2.3 fold higher INF-γ activity was induced. Moreover, in immunized mice treated with plant PS, the production of IL-2 was 1.5 to 3.9 fold higher, whereas IL-4 level increased 4.6 to 6.4 fold higher as compared with the control group. These results indicate that one of the mechanisms regarding the stimulating effects of plant PSs to protective immunity of bacterial glycoconjugate immunogen is through the enhancing activities of cytokines and immune responses.

Application of plant PSs to bacterial glycoconjugate vaccines could provide more effective protective immunity for the prevention of pneumococcal infection.

Key words Herbal polysaccharides, Immunomodulating effects, Protective immunity, Pneumococcal glycoconjugate, Immunostimulating mechanism.

Introduction

Recently, immunomodulating and antitumor activities have been observed in the polysaccharide (PS) fractions of herbal products, such as gluconans and mannans isolated from the lentinan of *Lentinus edodes* (Hsiang-ku) and *Ganoderma tsugae* (Ling-chih).[1,2] Many of these PSs have profound effects on the immune system, and are relatively non-toxic. The mechanisms of these activities are considered to involve the functions related to amplification of phagocytes, activation of macrophages, T-lymphocytes, and alternative complement pathway and enhancement of the cell-mediated immune response.[3,4]

The immunity of the body decreases with age, causing an increased incidence in various diseases such as infections, arthritis, diabetes, and cancer in the elderly. Since immunomodulating PSs stimulate the immune response, they could promote the antimicrobial activity and prevention of diseases.[5,6]

New sources of biochemically active PSs are drawing great attention, and the demands for immunostimulating agents for the treatments of various immunodeficient diseases are increasing. Juzen-taiho-to (十全大補湯 ; Shi-Quan-Da-Bu-Tang), a dried

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decoction of 10 herbal mixture contains *Astragali Radix, Cnidii Rhizoma, Angelicae Radix, Hoelen,* and several other herbal products. In Japan, it has been observed to exhibit reduction of side effects for mitomycin C, enhancement of anti-tumor activity, and activation of C3 complement and macrophages. In addition, it stimulates the immune response against sheep red blood cell antigens, cellular immunity of T-lymphocytes, and delayed-type hypersensitivity.[7-10] However, the immunologic activities of PS components contained in these herbal products and their function mechanisms have not been extensively studied. Present studies report the preparation of PSs from *Angelica Radix* and *Hoelen* and their immunomodulating activities.

Materials and Methods

Materials : *Angelicae Radix* (Ibaraki, Japan) and *Hoelen* (An-Huei, China) were provided by Central Research Loboratories, Tsumura & Co., Ibaraki, Japan. Pneumococcal type 9V PS conjugated with inactivated pneumolysin (ply) was prepared by laboratory of Center for Biologics Evaluation and Research, FDA, Bethesda, MD, U.S.A.[11] Alkaline phosphatase-rabbit anti-mouse IgG and IgM conjugates, p-nitrophenyl phosphate, thioglycollate, concanavalin A were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Balb/c mice were obtained from Small Animal Production Section, National Institutes of Health, Bethesda, MD. RPMI medium 1640, Hank's balanced salt solution (HBSS) were purchased from Gibco BRL, Grand Island, N.Y. Sephadex G-150, Sephadex G-50, and DEAE-Sephadex A-50 were purchased from Pharmacia, Piscataway, N.J. Mouse Interferon-γ ELISA kit, mouse TNF-α ELISA kit and mouse IL-2, IL-4, IL-6 ELISA kits were obtained from Endogen Inc., Woburn, MA. Lentinan was obtained from Ajinomoto, Co., Tokyo, Japan.

Preparation of polysaccharides : Dried herbal product, 1 kg, was refluxed and extracted with methanol, 10 liters, for 2 hrs. The methanol-insoluble fractions were refluxed with distilled water, 10 liters, for 2 hrs. The aqueous extract was concentrated under vacuum with a Rotavapor to a volume of 150-200 ml. Crude

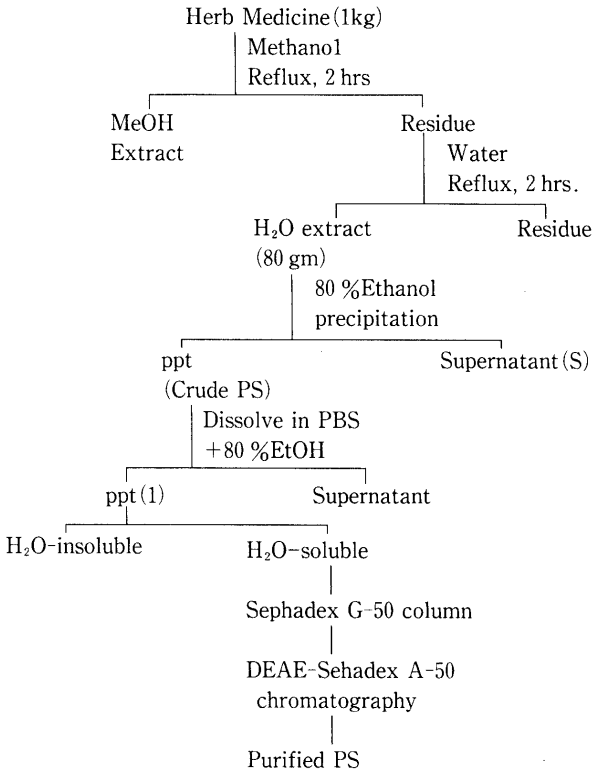

Figure 1 Isolation and purification of plant PSs.

polysaccharides were precipitated with adjusting the solution to 80 % ethanol concentration. The precipitate was centrifuged at 10,000×g for 15 min and dissolved in 50 ml of phosphate buffered saline (PBS), pH 7.4. The process of ethanol precipitation was repeated. The precipitate was dissolved in 50 ml of distilled water. The soluble fraction was passed through Sephadex G-50 column, 1.5×90 cm, and eluted with distilled water. PS fractions were detected by phenol-sulfuric acid method (5 % reagent, measuring optical density at A_{480nm}).[12] Pooled PS fraction was further purified by DEAE Sephadex A-50 column, 1.5×90 cm and eluted with a concentration gradient of 50 mM to 1 M sodium phosphate buffer, pH 8.0. PS fractions were dialyzed against distilled water and lyophilized (Figure 1). This PS was further purified with phenol-chloroform extraction. The aqueous fraction was separated and repeated the extraction three times. The fraction was dialyzed against distilled water and ultracentrifuged at 100,000×g for 3 hrs and used for experiments.

Determination of molecular weight : Gel filtration on a 1.5×90 cm column of Sephadex G-100 was used

to determine the average molecular weights of the PSs (5 mg/ml) using PBS, pH 7.4 as the eluent. The elution volume was collected in 2 ml fractions. Phenol-sulfuric acid method was used for determination of PSs. A calibration curve was constructed by using dextrans with known molecular weights (14.8, 23.8, 48.6, 66.8, and 78 KD, Fruka Chemie AG, Buchs, Switzerland). The average molecular weight of PS was determined by using the calibration curve.

Analyses of sugar components by gas chromatography : The plant PS, 10 mg was hydrolyzed with 2 N trifluoroacetic acid at 121°C for 1 hr, followed by reduction with sodium borohydride. The resulting alditols and aldonic acids derived from aldoses and uronic acids respectively were separated by anion-exchange resin chromatography using AG1-X2 resin, acetate form (Rio-Rad). The alditols were acetylated with acetic anhydride at 121°C for 3 hrs[29,32] and analyzed by a Hewlett-Packard model 5890a capillary gas chromatography (GC) system equipped with a DB-WAX capillary column, 0.25 mm i.d.×30 cm, 0.25 um-film thickness (J & W Scientific), an integrator (HP3393A +HP9122C), and an auto-sampler (HP7673A). The aldonic acids were reduced with sodium borohydride again into the corresponding alditols and then acetylated with acetic anhydride.[30,33] The resulting alditol-acetates were analyzed by GC in the same manner. Following conditions were used for GC analysis : injection, 1.0 μl (splitless, 240°C) ; detection, FID (250°C) ; carrier gas, helium (15 psi) ; column temperature-program−after injection, 120°C was kept for 5 min and then rised up (5°C/min, linearly) to 240°C and kept at 240°C for 35 min. The peaks were identified by comparing the retention times with those of alditol acetate from authentic sugars.[13]

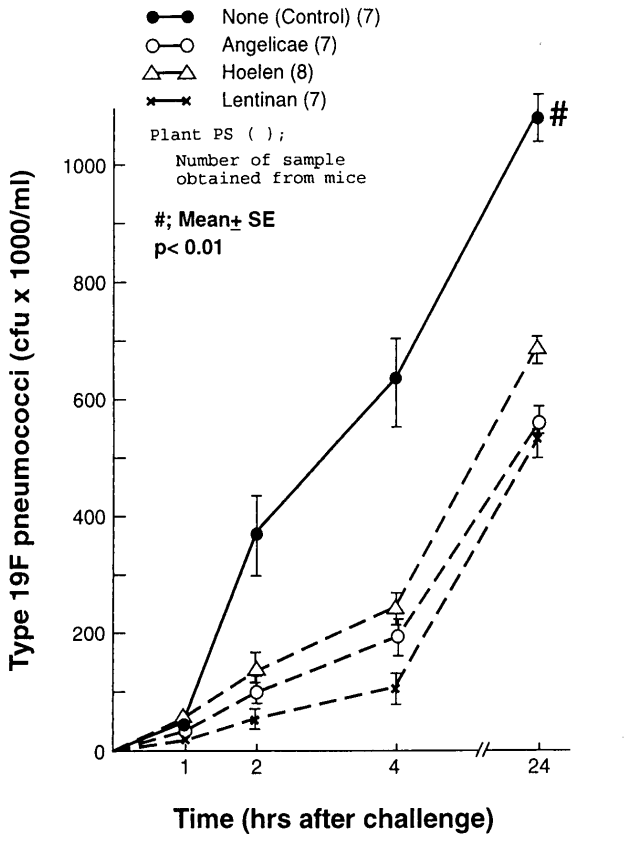

Figure 2 Bacterial clearance of mice treated with plant PS.

Effects of herbal PSs on immune responses to pneumococcal type 9V PS-ply conjugate : Balb/c mice, 8-10 weeks of age, 5-8 mice per each group were injected i.p. with plant PS 0.2 mg or 1.0 mg/0.1 ml saline at day 1 and day 2. Pneumococcal type 9V PS-ply conjugate, 5 μg containing 50 μg aluminum hydroxide adjuvant/0.1 ml saline was injected i.p. at day 3 and day 10. At 7 days after the final injection blood samples were collected and the serum levels of 9V PS IgG and IgM antibodies were determined by ELISA.[14,15]

Bacterial clearance : Groups of 8-week old female mice, 7 to 8 mice in each group were injected i.p. with the 0.2 mg/dose of plant PSs, once per day, twice. Four hrs after the second injection, the mice were challenged with type 19F pneumococci 1.2×10^5 cfu/0.1 ml i.p. At 1, 2, 4, and 24 hrs after injection, 20 μl blood samples were collected from a tail vein, dispersed in 100 μl medium and innoculated immediately on sheep blood agar plate. Colonies were counted after overnight incubation at 37°C.

Determination of cytokine inducing activities : Balb/c mice, 8-10 weeks of age, were treated with type 9V PS-ply conjugate 5 μg/dose once or twice at 10 days interval between injection. Seven days after the final injection, the mice were injected i.p. with 3 ml of 3 % thioglycollate medium to induce macrophage and killed 4 days later. Macrophages were collected, washed with cold PBS and resuspended in RPMI 1640 medium (10^6 cells/ml). Mouse (Balb/c strain) spleen was removed, washed with Hank's balanced salt solution (HBSS). Spleen cells were washed with HBSS

and resuspended in RPMI 1640 medium containing 10 % fetal calf serum (FCS), 100 IU/ml penicillin and 100 μg/ml streptomycin.

Nine parts of spleen lymphocyte suspension were mixed with 1 part of peritoneal macrophage suspension. The mixed suspension was used in the interferon induction experiment. Plant PS was dissolved in RPMI 1640 medium and added to mixed cell suspension (0.2 mg/ml). The cells were incubated at 37°C with 5 % carbon dioxide for 72 hrs. Viability of cells was measured by trypan blue exclusion method. The cells were centrifuged at 1,500×g for 20 min. Productions of interferon-γ and TNF-α were determined in the supernatant by ELISA.[16,17]

Determination of interleukins : Balb/c mice, 8-10 weeks of age, 5 to 8 in each group were injected i.p. with plant PS, 0.2 mg/0.1 ml in saline. Three days later, pneumococcal type 9V PS-ply conjugate, 5 μg/0.1 ml containing adjuvant, was injected i.p., once per 10 days, twice. Seven days after last injection, spleen cells from plant PS-treated and non-treated mice were prepared and suspended in PRMI medium 1640 containing 10 % fetal calf serum, penicillin (100 IU/ml) and streptomycin (100 μg/ml). Aliquots, 0.1 ml of cell suspension (2.5×10^6 cells/ml) were added to Con A (10 μg/ml) in the medium containing 10 % FCS and incubated at 37°C for 72 hrs. The cell suspension was centrifuged and the supernatant was determined for production of interleukins IL-2, IL-4 and IL-6.[18-20]

Results

Physicochemical properties of plant PSs

The GC analyses revealed that the Angelica PS consisted of glucose, galactose, arabinose and rhamnose as major neutral sugars and galacturonic acid as acidic component. Other sugar contents including fucose, and xylose were also detected. Hoelen PS consisted mainly of neutral sugars including galactose, glucose, mannose, and fucose. Galacturonic

Table I Effect of plant PS on antibody response to pneumococcal type 9V PS glycoconjugate.

Treatment		Antibody response		
9V conj (5μg)	Plant PS (mg)	Angelica	Hoelen	Lentinan
		(9V IgG Ab/ml serum)		
(I)				
+	−	1.92±0.92# (5)		
+	0.2	*4.14±0.80 (7)	*2.97±0.34 (6)	ND
+	1.0	**4.62±0.63 (7)	**5.24±0.91 (5)	ND
(II)				
+	−	1.83±0.27 (7)		(0.1 mg)
+	1.0	**4.33±0.64 (6)	**6.35±0.95 (7)	*2.99±0.54 (6)
		(9V IgM Ab/ml serum)		
+	−	1.68±0.31 (6)		
+	1.0	**3.88±0.43 (8)	**4.01±0.29 (8)	**4.65±0.29 (6)

Experiment I (used 0.2 and 1.0 mg plant PS) and II (used 1.0 mg plant PS and also measured 9V IgM Ab response) were performed at different time.
: Mean±standard error.
( ) : Number of mice used in the experiment.
* : $p<0.05$, ** : $p<0.01$ when mice treated with plant PS and 9V conjugate were compared with the group that treated with 9V conjugate alone.
ND : Not determined.

acid was a major acidic component. From 1 kg of dried plant material the yields of 404 mg purified Angelica PS and 394 mg Hoelen PS were obtained.

Molecular weights of Angelica and Hoelen PSs were 61,000 and 21,000 respectively as determined by gel-filtration. Molecular weights of these plant PSs were much smaller than that observed in bacterial PSs (>200,000). During isolation procedure plant materials were treated with methanol reflux for 2 hrs followed by water reflux for 2 hrs. After such treatment, the PSs contained in herbs, such as *Astragalus Radix* or *Cnidii Rhizoma* exhibited few degraded peaks when passed through gel filtration column (Lee and Koizumi, unpublished observations). Certain plant PSs appear to be sensitive to heat treatment and may degrade. It is more appropriate to extract plant PSs at 25°C or lower temperature.

Effects of plant PSs on immune response to pneumococcal type 9V PS-ply conjugate

Enhancement of antibody response to pneumococcal 9V PS-ply conjugate is shown in Table I. The mice treated with Angelica or Hoelen PS followed by administration of pneumococcal type 9V PS-ply conjugate induced higher serum levels of 9V PS IgG and IgM antibodies than the non-treated control group.

Bacterial clearance of mice injected with plant PSs

Mice treated with plant PSs were challenged i.p. with type 19F pneumococci to determine the effectiveness of bacterial clearance from the blood (Figure 2). Mice injected with Angelica or Hoelen PS prior to challenging with 1.2×10^5 cfu type 19F pneumococci showed more rapid bacterial clearance from blood 2, 4, and 24 hrs after challenge as compared to the non-treated control group.

Cytokine inducing activity

Table II shows cytokine inducing activity of plant PSs in mice immunized with type 9V PS-ply conjugate. In immunized mice treated with Angelica or Hoelen PS, 0.2 mg/ml, the inducing activity of TNF-α from macrophage and spleen cells showed 4.0 to 5.5 fold higher than the non-treated control group. The inducing activity of TNF-α caused by treatment with these PS under present experimental condition was similar to the stimulating effect caused by Lentinan. In experimental mice given the second dose of 9V PS conjugate, their inducing activity of TNF-α showed 2.4 to 4.6 fold increase as compared with the control group.

In immunized mice treated with Hoelen PS, the inducing activity of INF-γ showed 2.3 fold higher than the control group. However, Angelica PS did not show significant effect on induction of INF-γ.

Production of interleukins

Table III shows the production of IL-2, IL-4 and IL-6 by the plant PSs in mice immunized with type 9V PS-ply conjugate. In immunized mice treated with Angelica or Hoelen PS, 1 mg/dose once or twice, production of IL-2 was 1.5 to 3.9 fold higher than that of non-treated control group, whereas IL-4 level increased 4.6 to 6.4 fold as compared with the controls. In contrast, the effect of plant PSs on production of IL-6 was not observed.

Table II Cytokine induction by plant PS in mice immunized with pneumococcal type 9V PS glycoconjugate.

	Treatment	Plant PS (0.2 mg)			
	Immunized 9V conj	None	Angelica	Hoelen	Lentinan
TNF-α (μg/ml)	5 μg	2.4 ±0.78# (4)	** 9.7±1.65 (4)	**11.3±1.61 (4)	**7.70±0.53 (4)
	5 μg×2	5.35±2.52 (4)	**24.5±4.33 (6)	**13.1±1.57 (6)	**17.7±3.63 (7)
INF-γ (μg/ml)	5 μg	4.63±1.23 (6)	5.20±0.99 (6)	**10.8±1.66 (6)	**18.6±1.41 (7)

: Mean±standard error.
( ) : Number of samples.
** : $p<0.01$ when immunized mice treated with plant PS were compared with the group that with immunized with 9V conjugate alone

Table III Interleukin stimulation by plant PS in mice immunized with pneumococcal type 9V PS glycoconjugate.

Treatment		Angelica	Hoelen	Lentinan
9V conj. ($5\ \mu g$) twice	Plant PS (1 mg)			
	IL-2	(μg/ml)		
+	−	6.64±2.45 (5)		
+	+	**25.5±5.26 (7)	*10.4±2.47 (6)	**25.5±5.65 (4)
	IL-6	(μg/ml)		
+	−	17.3±2.17 (8)		
+	+	19.4±2.80 (7)	18.4±2.35 (8)	19.5±2.39 (8)
	IL-4			
+	−	1.43±0.17 (6)		
+	+	**9.88±2.35 (6)	*6.67±1.98 (6)	**8.88±2.34 (5)

: Mean±standard error.
( ) : Number of samples.
* : $p<0.05$
** : $p<0.01$ when mice treated with plant PS and 9V conjugate were compared with the group that treated with 9V conjugate alone.

Discussion

Herbal products (Kampo drugs, Chinese traditional medicines) have been used in east Asia for many years and attracted attention recently in many areas of the world. Some of these compounds exhibit promise as possible therapeutic agents for certain diseases, such as allergy, cancer, AIDS and chronic degenerating diseases.[21] The mechanisms of the immunostimulation and antitumor activities of plant products are thought to involve the functions related to amplification of phagocytosis, activation of macrophages, T-lymphocytes, alternative complement pathway and enhancement of the cell-mediated immune response. When homeostasis of physiological condition is changed due to environmental stress or disease, the functions of immune system are decreased or suppressed. Such effects are usually observed in clinical symptoms of low eating appetite, weakness of body, anemia and decrease of host defense activity to infection. For treatment of these clinical symptoms, certain herbal formula such as Juzen-taiho-to, Ninjin-yoei-to, and Seinetsu-hoki-to have been used to enhance the immune fuctions. These herbal products contain various components of polysaccharides.

The results of present studies revealed that Angelica and Hoelen polysaccharides contained in several herbal formula enhanced the PS IgG and IgM antibody responses to pneumococcal type 9V PS-protein conjugate, and caused rapid bacterial clearance from blood after challenge with virulent pneumococci. These plant PSs also stimulated induction of cytokine activities of TNF-α, INF-γ as well as production of interleukins IL-2 and IL-4. The purified plant PSs contained less than 0.1 endotoxin unit/mg PS as measured by Limulus amebocyte lysate assay[31] and would not affect their activities of cytokine induction. These results suggest that one of the mechanisms regarding the stimulating effects of plant PSs to protective immunity of bacterial glycoconjugate immunogen is through the enhancing activities of cytokines and immune responses. Application of plant PSs to bacterial glycoconjugate vaccines may provide more effective immunization for the prevention of pneumococcal infection.

Juzen-taiho-to (TJ-48, Shi-Quan-Da-Bu-Tang) has been used to support the immune functions and

promote general health of the patients recovering from surgery or suffering from chronic diseases. It has been reported to exhibit profound effects in enhancing immune responses including antibody production to sheep blood cell antigen,[7,22] phagocytosis[23] mitogenicity and complement-activating activity. Among various fractions of TJ-48, only the fraction containing pectic polysaccharides was found to have both mitogenicity and complement-activating activities.[10,24] These activities might be caused by the additive effects of several herbal components in TJ-48. The results of present studies provide additional evidence that the polysacchrides in TJ-48 component such as *Angelica* and *Hoelen* enhanced the protective immunity to bacterial PS-protein conjugate immunogen and stimulated induction of cytokine activities.

Angelicae Radix has been used in therapies for gynecological diseases and arthritis in Japan. The polysaccharide fraction of *Angelica* prepared from a water extract has been reported to exhibit various activities of complement-activation, mitogenicity to B-lymphocytes, interferon-production, and anti-tumor to Earlich ascites.[25-28] These activities were distributed in different structural components of polysaccharide. The complement activation by its active component, AR-arabinogalactan was through both the alternative and the classical pathway. The Angelica PS prepared in present study contained sugar uronic acid, rhamnose, fucose, and xylulose in addition to arabinose, galactose and glucose which were main sugars in arabinogalactose. The molecular structure of these sugar components may form certain immunologic epitope that involed in inducing the immunostimulating effects. The relationship between the complement-activation, protective immunity of pneumococcal glycoconjugate to bacterial infection and cytokine induction activities of Angelica PS to the backbone and tertiary structure of the PS molecule needs to be investigated further.

There are a number of polysaccharides contained in various herbal products, such as Juzen-taiho-to which may contain pharmacologically-active components for useful therapeutic agents. Thus, further research can also be performed in immunochemical characterization of plant PSs contained in TJ-48, such as *Astragali, Cnidii etc.*, and their structure-immunomodulating activity relationships.

It has been applied for many years that the herbal products have used complex formula including more than two kinds of herbs. However, it is still not clear what mechanisms involve that the traditional herbal medicines should be used as complex formula. It is necessary to elucidate what is the scientific basis for the combined product formula on the enhancing immunostimulating effects and pharmacological activities.

In addition, it has been realized that effective scientific approaches in evaluation of plant products could serve as the basis for the acceptance and approval of herbal products by the Western medical community and regulatory agencies.

和文抄録

生薬，当帰および茯苓から多糖類を分離して，これらの免疫調節作用について検討した。

当帰由来の多糖はグルコース，ガラクトース，ラムノース，アラビノース，ガラクツロン酸などの成分であり，茯苓はグルコース，ガラクトース，マンノース，ガラクツロン酸などであった。

これらの生薬由来多糖類をそれぞれのマウスに投与し，その肺炎球菌 9V 型多糖類蛋白質結合抗原を接種したところ，投与しない対照群に比べて，より高い 9V 型多糖類 IgG および IgM 抗体を産生した。

生薬由来多糖類をそれぞれ投与したマウスに，肺炎球菌 19F 型をチャレンジしたところ，血中からの迅速な抗菌作用がみられた。

さらに，9V 型多糖類蛋白質結合抗原を接種した免疫マウス群の脾臓細胞に，生薬由来多糖類を作用させたところ，対照群に比べて，TNF-α の産生が 4.0～5.5 倍，IL-2 は 1.5～3.9 倍，IL-4 は 4.6～6.4 倍に高く誘導され，茯苓についてはこの他に INF-γ が 2.3 倍誘導された。

これらの結果は，細菌性多糖類蛋白質結合抗原の防御免疫において，生薬由来多糖類がサイトカインと免疫応答のメカニズムに関与しているものと考えられる。

また，細菌性多糖類結合ワクチン接種に際して，生薬由来多糖類の利用は，肺炎球菌感染に対するより効果的な防御免疫作用を提供するものといえよう。

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