Polysaccharide of *Astragali Radix* Enhances IgM Antibody Production in Aged Mice

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Received June 30, 1997; accepted August 20, 1997

The effect of *Astragali Radix* (AR) on IgM antibody production in mice of various ages (10 weeks, 36 weeks and 60 weeks) was examined. The antibody production levels in the 36- and 60-week-old mice were significantly decreased to about 70 and 60% of that in the 10-week-old mice. The enhancement effect of a crude polysaccharide AR fraction on the antibody production was nil in the 10-week-old mice, but significant enhancement effects were observed in the 36- and 60-week-old mice, compared to the age-matched control.

Two polysaccharides active in the enhancement of the IgM antibody production in the aged mice were isolated from the high molecular weight fraction of AR by cetavlon precipitation, ion-exchange and gel permeation chromatography. The molecular masses of these polysaccharides were calculated by HPLC in salt solution. Only one major peak was observed for each, and their molecular masses were estimated to be 1.2×10^4 and 2.2×10^4 . The major components of these polysaccharides were neutral carbohydrates (89.3 and 95.5%), followed by uronic acid and protein; glucose was the predominant sugar component.

Key words *Astragali Radix*; IgM antibody production; aged mouse; polysaccharide; oral administration

With aging the human body's defensive ability weakens,¹⁾ including aspects of antibody production,²⁾ the production of cytotoxic T-cells³⁾ and cellular immune function.⁴⁾ *Astragali Radix* (AR) is an important traditional Chinese herbal medicine widely used in China and Japan. It is clinically used in China as an invigorating drug to improve a reduced immune response, often for the elderly.⁵⁾

We recently reported a defensive effect of AR in young mice (5—12 weeks), including its protective effect against Japanese encephalitis virus infection,⁶⁾ the enhancement of antibody production by mouse spleen cells,⁷⁾ and a beneficial effect on the function of peritoneal exudate cells.⁸⁾ However, the active substances in AR have not yet been identified.

In the present study, we examined the effects of the oral administration of AR on IgM antibody production in mice of various ages as a measure of the protective ability of the medicine. We also purified the ingredients responsible for the antibody production enhancement and examined some of their chemical properties.

MATERIALS AND METHODS

Extraction and Fractionation of AR The samples of AR used were cultivated in Hokkaido. Each AR fraction was prepared as described in Fig. 1. Briefly, powdered AR (200 g) was treated with distilled water (1000 ml) in a water bath at 80 °C for 2 h. After filtration, the solution was concentrated to one-fifth its original quantity under reduced pressure at 40—50 °C, and precipitated by the addition of methanol (2000 ml). The resulting precipitate was dialyzed against distilled water for 3 d (regenerated cellulose tube, molecular weight cut-off: 3.5 kD, Spectrum Medicinal Industries Inc., CA, U.S.A.). The non-dialyzed fraction was collected and freeze-dried to give a crude polysaccharide fraction (F-1, 11.72 g). F-1 was further

separated by Yamada's method with slight modification.⁹⁾ In brief, to an aqueous solution containing 5% of F-1, an equal volume of an 8% aqueous solution of cetyltrimethylammonium bromide and 1/9 volumes of 0.1% sodium sulfate were added and the mixture was left standing overnight at 20 °C. The mixture was then centrifuged (3000 rpm, 10 min) to obtain the precipitate and supernatant. The precipitate was dissolved in 10% aqueous NaCl and reprecipitated with 3 volumes of methanol with 1% KOAc. The resulting precipitate was dialyzed against distilled water. The non-dialyzable fraction was a strongly acidic polysaccharide fraction (F-2, 3.56 g). One percent of boric acid was added to the supernatant, and the pH was adjusted to 8.8 with 2 N NaOH. After being stirred for 5 h, the solution was centrifuged (3000 rpm, 10 min), and the precipitate was treated by the same method as that used for F-2 to recover the weakly acidic polysaccharide fraction (F-3, 0.50 g). The remaining supernatant was acidified with acetic acid (pH 4.4) and 3 volumes of MeOH added with KOAc. The resulting precipitate was dialyzed, and a neutral polysaccharide fraction (F-4, 4.32 g) was obtained as the lyophilizate. F-2 (1 g) was dissolved in 0.02 N Tris-HCl buffer (pH 7.0) and applied to a column (7 cm × 35 cm) of DEAE-Sephacel (Pharmacia Biotech, Uppsala, Sweden), then eluted with the same buffer and 0—0.8 M NaCl (gradient). Fractions of 25 ml were collected and analyzed by the phenol-sulfuric acid¹⁰⁾ and Lowry methods¹¹⁾ (Fig. 4). F-2 was separated into 3 fractions (F-5, F-6, F-7). F-5 was obtained from tubes 10 to 13, F-6 from tubes 35 to 38 and F-7 from tubes 42 to 45. These fractions were lyophilized after dialysis. F-5 (50 mg) was dissolved in 0.1 M phosphate buffer (pH 7.2) containing 0.2 M NaCl and further separated by gel chromatography using a column (2.6 cm × 60 cm) of Sephacryl S-300 (Pharmacia) with the same solution. Fractions of 10 ml were collected and analyzed by the phenol-sulfuric acid method (Fig. 5). F-5

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was separated into F-8 and F-9. F-8 was obtained from tubes 22 to 28 and F-9 from tubes 29 to 34. These fractions were lyophilized after dialysis. Figure 1 shows the yields of these fractions from AR.

Chemical and HPLC Analysis The total carbohydrate content was determined by the phenol-sulfuric acid method⁽¹⁰⁾ using glucose (Glu) as a standard. The uronic acid content was determined by the *m*-hydroxybiphenyl method⁽¹²⁾ using galacturonic acid (GalA) as a standard, and protein contents were determined by the Lowry method⁽¹¹⁾ using bovine serum albumin as a standard.

Determination of component sugars was made by hydrolyzing a sample (2mg) with 2N trifluoroacetic acid at 100°C for 6 h. Neutral sugars were analyzed by gas chromatography (GC) after conversion of the hydrolyzate into alditol acetates using the routine method. GC was carried out using a Hewlett Packard 5890 II instrument equipped with a flame ionization detector and a fused silica capillary column of DB-23 (0.25 mm i.d. × 30 m, J&W Scientific, CA, U.S.A.) with a nitrogen flow rate of 1.2 ml/min. The initial column temperature was 180°C and 2 min later was elevated to 250°C in steps of 2°C/min. 2-Deoxy-D-glucose was used as an internal standard.

For HPLC analysis, F-8 and F-9 were dissolved in 0.1 M NaCl and filtrate (0.45 μm). The filtrate solution was applied to columns of Shodex Asahipak GF-510HQ, GF-310HQ (7.8 × 25 cm, Showa Denko, Tokyo, Japan) in series. A mobile phase of 0.1 M NaCl was used at the flow rate of 0.5 ml/min. The eluents were monitored by a differential refractometer. The molecular weight was calibrated with standard dextrans (Showa Denko); molecular weight = 5, 10, 20, 50, 100 and 200 × 10³.

Effect of AR on IgM Antibody Production Female C3H/HeN mice were obtained from Japan SLC Inc. (Shizuoka, Japan), and used at 10, 30, 36 and 60 weeks of age. They were acclimatized to their housing for at least 14 d prior to the experiment. The AR fraction was

dissolved in phosphate-buffered saline (PBS) and orally administered to the mice (0.1 ml/mouse) through a gastric tube once a day for 5 d. Age-matched control mice were administered PBS alone. The day after the final oral administration, the mice received an intravenous inoculation of the antigen dinitrophenylated dextran (DNP-dextran, 200 μg). After 5 d, blood samples for antibody determination were collected, and the serum was collected in a routine manner. The concentration of anti-DNP antibodies in the serum was determined by ELISA.

Determination of Antibody Concentration by ELISA ELISA was performed as reported earlier.⁽⁷⁾ In brief, to each well of a 96-well microplate was added 100 μl of DNP bovine serum albumin (DNP-BSA) dissolved in coating buffer (2 μg/ml), and the plate was incubated for 90 min at 37°C. The wells were then washed with PBS containing 0.5% Tween 20. The test solutions were diluted with PBS, 100 μl of which was added to each well, and the plate was left at 4°C overnight. After another washing, 100 μl of horseradish peroxidase-conjugated rabbit anti-mouse IgM diluted in PBS (×2000) was added to each well. The plate was incubated at 37°C for 1 h and washed. Then to each well was added 100 μl of a substrate solution. After incubation at room temperature in the dark for 30 min, 100 μl of 2N H₂SO₄ was added and the plate was read at 490 nm on a microplate autoreader. The concentrations were obtained by interpolation on the standard curves. The final concentration of IgM in each sample was calculated as the mean value of duplicate.

Statistical Analysis All data were analyzed by one-way analysis of variance (ANOVA) and the multiple comparison test (Fisher's PLSD test). If the *p*-value was below 0.05, we considered the difference significant.

RESULTS

Effect of F-1 on IgM Antibody Production in Mice of Various Ages As shown in Fig. 2, the IgM antibody production in the 36- and 60-week-old mice were significantly decreased, to levels about 70 and 60% of that in the 10-week-old mice. The enhancement effect of the crude polysaccharide AR fraction, F-1, on the IgM antibody production was nil in the 10-week-old mice

Fig. 2. IgM Antibody Production in Mice at 10, 36 and 60 weeks of Age

Each value represents the mean ± S.D. of five mice. ** Significantly different value at *p* < 0.01.

Fig. 1. Separation of Polysaccharide Fraction of *Astragali Radix*

Fig. 3. Effect of Astragali Radix Crude Fraction (F-1) on IgM Antibody Production in Mice of Various Ages

□, 10 weeks; ▨, 36 weeks; ■, 60 weeks. Each value represents the mean $\pm$ S.D. of five mice. *, ** Significantly different from age-matched control value, at $p < 0.05$, $p < 0.01$.

Table 1. Effect of *Astragali Radix* Polysaccharide Fractions on IgM Antibody Production

Sample	Yield from <i>Astragali Radix</i> (%)	Dose (mg/mouse)	Antibody Production ^{a)} (% of control)
F-1	5.86	1.2	134.6 $\pm$ 19.1*
F-2	1.78	0.8	115.4 $\pm$ 18.2
F-2	1.78	0.4	137.7 $\pm$ 36.0*
F-3	0.25	0.2	102.2 $\pm$ 20.6
F-3	0.25	0.1	101.3 $\pm$ 25.1
F-4	2.16	0.8	110.8 $\pm$ 7.2
F-4	2.16	0.4	99.3 $\pm$ 25.2
F-2	1.78	0.6	125.6 $\pm$ 18.4
F-5	0.92	0.6	151.0 $\pm$ 22.9***
F-5	0.92	0.3	141.6 $\pm$ 33.4**
F-6	0.11	0.1	136.4 $\pm$ 11.7**
F-6	0.11	0.05	118.5 $\pm$ 18.0
F-7	0.32	0.2	126.4 $\pm$ 17.4*
F-7	0.32	0.1	131.8 $\pm$ 0.6*
F-5	0.92	0.3	141.0 $\pm$ 21.3**
F-8	0.32	0.1	153.6 $\pm$ 35.1**
F-9	0.42	0.1	160.3 $\pm$ 40.1**

a) Each value represents the mean $\pm$ S.D. of five mice. *, **, *** Significantly different from control value, at $p < 0.05$, $p < 0.01$, $p < 0.001$. 30-week-old mice were used for all experiments.

(Fig. 3). In contrast, F-1 significantly enhanced antibody production in the 36- and 60-week-old mice, compared to the age-matched controls. The enhancement effect was increased with the higher age.

Effect of AR Fractions on IgM Antibody Production The effects of the AR fractions on IgM antibody production are shown in Table 1. The active crude polysaccharide fraction, F-1, was separated into three fractions (F-2, F-3 and F-4) by the method using Cetavlon in small amounts of sodium sulfate.⁹⁾ A significant enhancement effect of the strong acidic fraction (F-2) was observed at a dose of 0.4 mg/mouse. The weakly acidic (F-3) and neutral (F-4) fractions had no effect. The active fraction, F-2, was further separated into three fractions by a column of DEAE-Sephacel (Fig. 4): the weakly adsorptive fraction (F-5), the strong adsorptive

Fig. 4. Chromatogram of F-2 on a DEAE-Sephacel Column

Each fraction was monitored by the phenol-sulfuric acid method (●) and Lowry method (○). Fraction numbers 10–13, 35–38 and 42–45 were collected, dialyzed, lyophilized and designated as F-5, F-6 and F-7, respectively. For details see Materials and Methods.

Fig. 5. Elution Profile of F-5 on Gel Permeation Chromatography

Each fraction was monitored by the phenol-sulfuric acid method (●). Fraction numbers 22–28 and 29–34 were collected, dialyzed, lyophilized and designated as F-8 and F-9, respectively. For details see Materials and Methods.

fraction (F-6) and the strongest adsorptive fraction (F-7). These fractions showed significant effects, and their effects were stronger than that of F-2 despite their lower doses. The active fraction, F-5, was further separated, using a column of Sephacryl S-300, into two fractions (F-8 and F-9) (Fig. 5). These fractions showed approximately equal significant enhancement effects on antibody production, and were the strongest among all of the separated AR fractions.

Chemical and HPLC Analysis of F-8 and F-9 Examination of the chemical properties of the active polysaccharides F-8 and F-9 showed that F-8 contained 89.3% carbohydrate as Glu and 7.2% uronic acid as GalA; F-9 contained 95.5% carbohydrate, 7.5% uronic acid. (Table 2) These polysaccharides contained no protein.

The fractions each gave a single peak on HPLC gel chromatography (retention time: 25.3 and 27.3 min) (Fig. 6), and their molecular masses were estimated to be 2.2×10^4 and 1.2×10^4 . The peak at 33.1 min in Fig. 6 was representative of the solvent. The sugar moiety of F-8 was composed of rhamnose, ribose, fucose, arabinose, xylose, mannose, galactose and Glu in molar ratios of 2:2:1:2:6:2:3:100 (Fig. 7). That of F-9 was composed of fucose, xylose and Glu in molar ratios of 1:2:100.

Table 2. Chemical Properties of F-8 and F-9

	F-8	F-9
Molecular weight	2.2×10^4	1.2×10^4
Total protein (as BSA) ^{a)}	N.D. ^{b)}	N.D.
Total carbohydrate (as Glu) ^{a)}	89.3%	95.5%
Total uronic acid (as GalA) ^{a)}	7.2%	7.5%
Component sugars (mol ratio)		
Rhamnose	2	N.D.
Ribose	2	N.D.
Fucose	1	1
Arabinose	2	N.D.
Xylose	6	2
Mannose	2	N.D.
Galactose	3	N.D.
Glucose	100	100

a) BSA, Bovine serum albumin; Glu, Glucose; GalA Galacturonic acid. b) Not detected.

Fig. 6. Gel Permeation HPLC Chromatogram of F-8 and F-9

F-8 and F-9 were applied to columns of Asahipak GF-510HQ and 310HQ in a series. A mobile phase of 0.1M NaCl was used at a flow rate of 0.5 ml. The peak at 33.1 min was represented as the solvent.

DISCUSSION

There have been several reports on the mechanism of the reduced protective responses in the elderly.¹⁻⁴⁾ However, very few clinical trials testing the activation of the reduced immune response as with advancing age have been performed.

In the present study, we examined the effect of AR on IgM antibody production as a measure of the medicine's protective ability. Significant decreases in the antibody production were observed in the 36- and 60-week-old mice, compared to that in 10-week-old mice. Our results demonstrated the enhancement effect of AR in elderly mice by oral administration, the reduced antibody productivity in 36- and 60-week-old mice were recovered to the level of that in 10-week-old mice.

In China, AR is used clinically as an invigorating drug to improve a reduced protective response,⁵⁾ e.g., for elderly men. The present findings suggest the validity of AR treatment.

Fig. 7. GC Chromatogram of Component Sugars in F-8 and F-9

Rha, rhamnose; Rib, ribose; Fuc, fucose; Ara, arabinose; Xyl, xylose; Man, mannose; Gal, galactose; Glu, glucose; I.S., internal standard (2-deoxy-D-glucose).

The mechanism of reduced antibody production in the elderly has been thought to involve the lowering of activity of helper T-cells,¹³⁾ reduction of thymus function,¹⁴⁾ decrease of division response in B-cells to the antigen-forming cells¹⁵⁾ and decreased activity of specific antibody production in B-cells.¹⁶⁾ DNP-dextran, which is a thymus-independent antigen, was used in the present studies, and AR is thought to have activated the reduced function of B-cells in the aged mice. The details of the mechanism underlying the effect of AR remain to be clarified, and investigation will be necessary.

There are several reports on the immunological activity of high molecular weight compounds such as glycoproteins and polysaccharides.¹⁷⁾ In the present study, two such compounds (F-8 and F-9) that enhanced the IgM antibody production in aged mice by oral administration were isolated from the crude polysaccharide AR fraction. They each gave a single peak on HPLC gel chromatography in salt solution. The major chemical component of these compounds was carbohydrate, and the predominant sugar component was Glu.

We speculate that the active ingredient in these two polysaccharides might consist of a glucan-chain moiety, and that this ingredient plays an important role in the enhancement of the antibody production when administered orally.

Glucan, with α -1,4 and α -1,6-linkage chains and designated as AG-1, was reported to be an immunologically active polysaccharide obtained from the water extract of the AR original plant, *Astragalus mongholicus* BUNGE.¹⁸⁾ The AR sample used in the present study was cultivated in Hokkaido, and is therefore believed to be derived from *Astragalus membranaceus* BUNGE.¹⁹⁾ The structural similar-

ity of these two polysaccharides is as yet unknown. The contribution of the glucan-chain moiety to the activities of AR in the present experiments is now being studied.

The molecular masses of the active fractions, F-8 and F-9, were calculated by HPLC gel chromatography. When 0.1 M NaCl was used as an eluant, only one major peak was observed for each fraction, and their molecular masses were estimated to be 1.2×10^4 and 2.2×10^4 . However, in the case of eluant in water, all elution patterns changed and their relative molecular masses decreased dramatically (data not shown). These results suggest that these two polysaccharides were able to form a molecular aggregate formation in salt solution, but not in water.

A relationship between polysaccharide molecular aggregation and biological activity has been reported.²⁰⁾ In addition, Yamaoka *et al.* reported that the polysaccharide fraction of the Chinese traditional medicine Shosaiko-to was active in the augmentation of natural killer activity by oral administration.²¹⁾ They suggested that chemical structure of the polysaccharide contributes to the expression of activity by oral administration, and that the molecular weight, which relates to absorption from the gut, may also be important for the expression of the activity. Further investigations of these polysaccharides will require a more detailed study of the relationship between the aggregation and the effect on antibody production by oral administration.

In conclusion, our results demonstrated that AR administered orally enhanced the production of IgM antibody in elderly mice. Two active polysaccharides were isolated from the AR water extract. They each gave a single peak on HPLC gel chromatography in salt solution, and their molecular masses were estimated to be 2.2×10^4 and 1.2×10^4 . The major sugar components of these fractions was glucose.

REFERENCES

- 1) Macedonian T., Petterson W. J., *J. Immunol.*, **93**, 886—896 (1964).
- 2) Makinodan T., James S. J., Inamizu T., Chang M. P., *Gerontology*, **30**, 279—289 (1984); Price G. B., Makinodan T., *J. Immunol.*, **108**, 403—412 (1972).
- 3) Shigemoto S., Kishimoto S., Yamamura Y., *J. Immunol.*, **115**, 307—309 (1975).
- 4) Kay M. M. B., *Clin. Immuno.*, **2**, 322—323 (1975).
- 5) Syouyaku Konwakai, "Syouyaku No Senpin To Hyouka", Osaka Syouyaku Kyokai, Osaka, 1993, p. 16—26.
- 6) Kajimura K., Takagi Y., Ueda N., Yamasaki K., Sakagami Y., Yokoyama H., Yoneda K., *Biol. Pharm. Bull.*, **19**, 855—859 (1996); *idem, ibid.*, **19**, 1166—1169 (1996).
- 7) Kajimura K., Takagi Y., Ueba N., Mimura M., Sakagami Y., Yokoyama H., Yoneda K., *Nat. Med.*, **51**, 45—49 (1997).
- 8) Kajimura K., Takagi Y., Mimura M., Nakano M., Sakagami Y., Yokoyama H., Hatakeyama Y., Kumagaya T., Yoneda K., *Nat. Med.*, **51**, 361—364 (1997).
- 9) Yamada H., Kiyohara K., Cyong J. C., Kojima Y., Kumazawa Y., Otsuka Y., *Planta Med.*, **50**, 434—439 (1984).
- 10) Dubois M., Gilles K. A., Hamilton J. K., Rebers P. A., Smith F., *Anal. Chem.*, **28**, 350—356 (1951).
- 11) Lowry O. H., Rosebrough N. J., Farr L., Randall R. J., *J. Biol. Chem.*, **193**, 265 (1951).
- 12) Blumenkrantz N., Asboe-Hansen G., *Anal. Biochem.*, **54**, 484—489 (1973).
- 13) Li M., Hanada K., Toichi E., Kurozumi M., Baba M., Takeda T., *Biomed. Gerontol.*, **16**, 184—185 (1992).
- 14) Hirokawa K., *Kansen-ensyo-meneki*, **18**, 181—194 (1988).
- 15) Kishimoto S., Tomino S., Inomata K., Kotegawa S., Saito T., Kuroki M., Mitsuya H., Hisamitsu S., *Clin. Immunol. Immunopathol.*, **25**, 1—10 (1982); Ennist D. L., Jones K. H., St. Pierre R. L., Whisler R. L., *J. Immunol.*, **136**, 99—105 (1986).
- 16) Delfraissy J. F., Galanaud P., Dormont J., Wallen C., *Clin. Exp. Immunol.*, **39**, 208—214 (1980); Pahwa S. G., Pahwa R. N., Good R. A., *J. Clin. Invest.*, **67**, 1094—1102 (1981).
- 17) Kiyohara K., Yamada H., *Carbohydr. Polymers*, **25**, 117—122 (1994); Matsumoto T., Yamada H., *J. Pharm. Pharmacol.*, **47**, 152—156 (1995); Sendl A., Mulinacci N., Vincieri F. F., Wagner H., *Phytochemistry*, **34**, 1357—1362 (1993); Matsumoto T., Cyong J. C., Kiyohara H., Matsui H., Abe A., Hirano M., Danbara H., Yamada H., *Int. J. Immunopharmac.*, **15**, 683—693 (1993).
- 18) Huang Q. S., Lu G. B., Chen Y., Guo J. H., Wang R. X., *Acta. Pharm. Sinica*, **3**, 200—206 (1982).
- 19) Anetai M., Katsura E., Minamiyama Y., Miura T., Kaneshima H., Yamagishi T., *Nat. Med.*, **49**, 284—287 (1995).
- 20) Gao Q. P., Kiyohara H., Cyong J. C., Yamada H., *Planta Med.*, **55**, 9—12 (1989); Kiyohara H., Yamada H., Cyong J. C., Otsuka Y., *J. Pharmacobio-Dyn.*, **9**, 339—346 (1983).
- 21) Yamaoka Y., Kawakita T., Kaneko M., Nomoto K., *Biol. Pharm. Bull.*, **18**, 846—849 (1995).