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The Effects of Plantago-mucilage A from the Seeds of *Plantago asiatica* on the Immune Responses in ICR Mice

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Effects of plantago-mucilage A (P-MA) on the immune responses were studied in ICR mice. Mice were divided into 4 groups (10 mice/group), and P-MA at doses of 7, 21 and 63 mg/kg were orally administered to mice once a day for 21 consecutive days. Mice were immunized and challenged with sheep red blood cells (SRBC). P-MA at 63 mg/kg/day significantly increased the body weight gain and the relative weights of spleen and thymus, as compared with those in controls. However, there were no significant effects on liver weight due to P-MA treatment. Plaque forming cells (PFC) and hemagglutination (HA) titers to SRBC were significantly enhanced in mice dosed at 21 and 63 mg/kg/day P-MA, as compared with those in controls. Delayed-type hypersensitivity (DTH) reaction to SRBC, phagocyte activity and circulating leukocyte were also significantly increased in mice dosed at 63 mg/kg/day P-MA. These results demonstrate that P-MA markedly enhances both humoral immune and allergic reaction to SRBC at concentrations which don't act on the relative weight of liver.

Key words : Plantago-mucilage A, Immune response, Mice

INTRODUCTION

Plantago-mucilage A (P-MA) is a substance isolated from the seeds of *Plantago asiatica* (Plantaginaceae) which is utilized for antiphlogistic, diuretic, antidiarrheic, antirheumatic, and cough medicine in Chinese medicine as well as in folk medicine (Yun and Chang, 1977; Chang and Yun, 1978; Ko and Lim, 1978).

The P-MA has a high content of mucous polysaccharide. This fraction forms a gel in water, and is classified as a mucilage. There have been reported a few investigations for chemical compositions of the seeds of *Plantago asiatica*. Among them, aucubin showed potent liver protective activity against the animal model of hepatitis induced by CCl_4 intoxication (Yun *et al.*, 1980). Chang *et al.* (1984) have found that aucubin also can protect against CCl_4 and α-amanitin-induced liver damages in rats, hardly accompanied with toxic actions. Toda *et al.* (1985) described that geniposidic acid in the methanol extract of seeds of this plant was found to be the most potent antioxidative component, which was superior to that of *dl*-α-tocopherol. Recently it was reported that P-MA has remarkable hypoglycemic activity (Tomoda *et al.*, 1987). Yamada *et al.* (1986) have found that P-MA enhances the activation of complement system, but no details with relation to immune response have been reported. In addition, we have demonstrated that squalene (Ahn and Kim, 1991, 1992) and extracts of some Chinese herbs with antioxidative activity, *e.g.*, of *Panax ginseng* (Ahn *et al.*, 1988a,b,c; Ahn and Lee, 1991) and garlic (Ahn *et al.*, 1989) have immunopotentiating effects in mice or rats. Then, P-MA is also expected to enhance immune response to SRBC, thereby having antioxidative activity of *Plantago asiatica*. However, little is known about the *in vivo* immunological effects of P-MA.

The present study was undertaken, therefore, to investigate the dose-response relationships for the immunological effects of P-MA in ICR mice.

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MATERIALS AND METHODS

Animals

Male ICR mice, 6 weeks of age, weighing 17-21 g, were used. Animals were housed individually in each cage and acclimatized for at least 7 days prior to the use. The cages were maintained at 23±2°C and 50-60% relative humidity throughout the whole experimental period. Mice were fed with animal chows (Jeil Ind. Ltd., Korea) and tap water *ad libitum* but deprived of animal chows for 16 hr prior to sacrifice.

Isolation of planto-mucilage A

The dried seeds (500 g, Korean commercial product) of *Plantago asiatica* were extracted twice with 0.2% sodium carbonate (3,000 ml each) under stirring at room temperature for 1 h each time as described by Tomoda *et al.* (1981). After centrifugation (6,000× g, 20 min), the extracts were combined and poured into five volumes of ethanol, then centrifuged. The precipitate was dissolved in water and treated with ethanol again. The treatment with ethanol was repeated three times. The final precipate was dissolved in water, followed by dialysis against running distilled water. Plantago-mucilage A (18 g; yield 3.6%) was obtained as a grayish-white powder after lyophilization. Its solution in water gave the high intrinsic viscosity value of 39.5 at 30°C.

Materials and treatment

P-MA was dissolved in saline. P-MA (7, 21 and 63 mg/kg, respectively) was orally administered to mice through a zonde once a day for 21 consecutive days. Control mice were given the corresponding volume of saline.

Lymphoid organ and body weights

Mice were sacrificed by cervical dislocation on the next day after the last P-MA treatment, and liver, spleen and thymus were collected and weighed. Lymphoid organ weight ratio to body weight was calculated for each of mice.

Antigen preparation

Sheep red blood cells (SRBC) collected from a single female sheep were kept at 4°C in sterile Alserver's solution (pH 6.1). SRBC were washed three times with phosphate-buffered saline (PBS; Gibco Lab. Co., Grand Island, N.Y., U.S.A., pH 7.4) after centrifugation at 400×g for 10 min and diluted to provide a desired concentration by hemacytometer count.

Immunization

All mice were immunized by intravenous (i.v.) injection of 0.1 ml of SRBC suspension (1×10^8 cells/ml) 4 days prior to each assays as described by Lake and Reed (1976). To assess the delayed-type hypersensitivity (DTH) reaction, separate groups of mice were challenged by subcutaneous (s.c.) injection of 0.05 ml of SRBC suspension (2×10^9 cells/ml) into their left hind footpad 4 days after immunization.

Preparation and inactivation of serum

The blood sample from each mouse was obtained from the carotid artery. The blood was allowed to clot in polyethylene tubes at 4°C for 30-60 min, and then centrifuged at 700×g for 20 min. The serum was withdrawn and heat-inactivated in polyethylene tubes at 56°C for 30 min.

Preparation of spleen cells

The spleen cells from each group of mice were washed three times by centrifugation and finally suspended in cold complete medium (RPMI-1640 medium supplemented with 100 unit penicillin/ml, 100 μg streptomycin, and 2 mM L-glutamine) as described by the modified method of Mishell *et al.* (1980). The cells were counted and the viability was determined by trypan blue exclusion test. Cell viabilities were never less than 95%.

Hemagglutination (HA) titers

HA titer was determined in microtitration trays (Limbro Chemical Co., Inc. New Haven, Connecticut, U.S.A.) using 0.025 ml volume of diluent by serial dilution of inactivated pooled sera in Hank's balanced salt solution (HBSS; Gibco Lab. Co., Grand Island, N.Y., U.S.A.) in plastic microtiter plate, which was added on to 0.05 ml volume of 0.5% packed SRBC as described by Yoshikai *et al.* (1979). The specified plate (Flow Lab., U.S.A.) was incubated for 18 hr at 37°C. Each titration was performed in duplicate and the mean titer was expressed as $\log_2$.

Assay of plaque forming cells (PFC)

In order to examine whether P-MA accelerates the antibody production to heterologous antigen or not, the slide technique of Cunningham and Szenberg (1968) was utilized. The number of direct PFC was counted 4 days after the immunization with 10^7 SRBC i.v. Numbers of PFC were expressed as those per 10^6 viable spleen cells or per spleen.

Assay of delayed-type hypersensitivity (DTH) reaction

Four days after immunization, mice was challenged s.c. in the left and right hind footpads with 10^8 SRBC and the corresponding volume of saline, respectively. The footpad swelling was evaluated by measuring the increase in thickness with a microcaliper (Mitutoyo Mfg. Co., Ltd., Japan) displayed in 0.01 mm gradation as described by Titus and Chiller (1981) and Henningsen *et al.* (1984). At 24 hr after challenge, the extent of swelling was calculated by subtracting the thickness of the saline-injected footpad from that of the antigen-injected footpad.

Assay of phagocyte activity

Phagocyte assay was determined by the modified method of Biozzi *et al.* (1954). In brief, for the preparation of colloidal carbon solution, rotring ink was diluted 1/6 with 1% gelatin and kept in a stoppered tube at 37°C during the experiment. In order to measure the phagocyte activity, separate groups of mice were challenged via the lateral tail vein by using a 1 ml syringe with 26 gauze needle at the dose of 0.01 ml of colloidal carbon solution per gram body weight of mouse. At the interval of 10, 20 and 30 min, 20 μl of blood sample was obtained from the retro-orbital venous plexus. The collected blood samples were expelled into each vial containing 2 ml of 0.1% sodium carbonate, and the contents were well mixed for the lysis of erythrocytes. The absorbance of the colloidal carbon contained in blood was measured directly with spectrophotometer (Bausch and Lomd, Sphen, U. S.A.) at 600 nm. Ten times of density readings were converted to logarithmic scale and plotted against time. The scope of the line is called phagocytic coefficient K. The mice were killed and the weights of spleen and liver were measured. Corrected phagocytic index which is a measure of phagocyte activity per unit weight of tissue. Corrected phagocytic index= body wt./(spleen wt.+liver wt)]× $\sqrt[3]{K}$.

Count of circulating leukocytes

Blood samples for measuring leukocytes in mice were collected from the retro-orbital plexus immediately before assay. Türk's solution was used for staining leukocytes and lysis of unnucleated cells. The number of nucleated cells was counted in hemacytometer chamber under a microscope. Triple counting per sample was carried out and the mean value of the results was calculated. The number was compared with that obtained from control mice.

Statistical analysis

Values are expressed as means±standard error (S. E.). All data were examined for their statistical significance of difference with Student's *t*-test.

RESULTS AND DISCUSSION

General response of mice to P-MA administration

There have not been investigated *in vivo* data regarding effects of P-MA on the immune system, although a number of pharmacological studies of *Plantago asiatica* have been published (Yun and Chang, 1977; Chang and Yun, 1978; Ko and Lim, 1978). Thus, we selected various doses from both our preliminary study of P-MA (data not shown) associated with immune response and the paper of *Plantago asiatica* methanol extract which have been previously reported as having hypotensive action in rabbits (Ko and Lim, 1978). Body and organ weights of mice dosed at 7, 21 and 63 mg/kg/day P-MA for 3 weeks are shown in Table I. There were no significant differences in liver weights by mice dosed at 7, 21 and 63 mg/kg/day P-MA compared with controls during the experiment. However, the body weight gain and the relative weights of spleen and thymus were significantly increased in mice dosed at 63 mg/kg/day P-MA compared with controls. In addition, all groups of mice were in good health with no clinical signs. These results, therefore, indicate that P-MA at doses used here did not cause any signs of toxicity in mice and are also expected to enhance immune responses, associated with enlargement of spleen and thymus.

Immune alterations

Antibody responses were performed to evaluate humoral immunity following p.o. treatment with P-MA using the T-dependent antigen, SRBC. Direct antibody plaque forming cells (PFC) response of spleen cells to SRBC after immunization was observed in mice given P-MA, and the enhancement in PFC per 10^6 spleen cells was statistically significant in mice dosed at 63 mg/kg/day P-MA (*i.e.*, 1,530±35, $p<0.05$), as compared with those in control mice (1,425±28). Their PFC per spleen were also increased significantly with an increased dose of P-MA (Table II). Hemagglutination (HA) titers of serum to SRBC were significantly enhanced in mice dosed at 21 and 63 mg/kg/day P-MA (*i.e.*, 6.49±0.15, $p<0.01$ and 6.22±0.14, $p<0.05$, respectively), as compared with those in control mice (5.56±0.20) (Table II). Yamada *et al.*

Table I. The effects of plantago-mucilage A on body and organ weights in ICR mice

P-MA dose (p.o.) (mg/kg/day)	Body wt. gain (%)	Percentage of body weight		
		Liver	Spleen	Thymus
0	68.64±1.29	4.51±0.06	0.48±0.02	0.13±0.01
7	63.44±3.38	4.55±0.06	0.46±0.03	0.14±0.00
21	72.86±3.28	4.41±0.07	0.53±0.01*	0.13±0.01
63	78.11±3.81*[a)]	4.63±0.08	0.63±0.04**	0.17±0.01*

Abbreviations: plantago-mucilage A, P-MA. P-MA (7, 21 and 63 mg/kg, respectively) were orally administered to ICR mice daily for 21 consecutive days. Mice were immunized i.v. with 10^7 SRBC 4 days prior to each measurement. Each value represents the mean±S.E. of 10 mice. *a)* Asterisks denote a significant difference compared with the value in control mice that did not receive P-MA. *$P<0.05$, **$P<0.01$

Table II. The effects of plantago-mucilage A on antibody responses in ICR mice

P-MA dose (p.o.) (mg/kg/day)	PFC/10^6 spleen cells	PFC/spleen ($\times 10^5$)	HA titer ($\log_2$)
0	1,425±28	2.46±0.09	5.56±0.20
7	1,401±72	2.31±0.10	5.48±0.25
21	1,535±58	2.89±0.16*	6.49±0.15**
63	1,530±35*[a]	3.01±0.11**	6.22±0.14*

Abbreviations: plantago-mucilage A, P-MA; plaque forming cells, PFC; hemagglutination, HA. Mice were immunized i.v. with 10^7 SRBC 4 days prior to each assay. Each value represents the mean±S.E. of 10 mice. [a]Asterisks denote a significant difference compared with the value in control mice that did not receive P-MA. *P<0.05, **P<0.01

(1986) reported that P-MA activates complement via the alternative pathway which is directly activated by certain immunoglobulins, polysaccharides, and certain animal cells. We have shown that the enhancement of the primary antibody response by P-MA was not due to T cells and accessory cells but due to B cells. Thus, these findings suggest that immunopotentiating effects by P-MA may be caused primarily by the activated function and the increase in number of B-cell populations. It seems plausible that P-MA may also enhance either direct or indirect activation of complement as discussed by Yamada *et al.* (1985). The precise mechanism for the enhancement of antibody response by P-MA remains to be investigated further.

Delayed-type hypersensitivity (DTH) reaction to SRBC was utilized to evaluate cell-mediated immunity following *in vivo* P-MA treatment. Table III shows the effects of P-MA on DTH response in ICR mice. The DTH reaction on footpad swelling were significantly increased in mice dosed at 63 mg/kg/day P-MA (*i.e.*, 1.92±0.10 mm at the dose of 63 mg/kg/day, p<0.05 as compared with 1.60±0.10 mm in controls). It has been reported that at least two separate subpopulations of T helper (Th) cells exist in the mouse, Th 1 cells, which secrete interleukin-2 (IL-2) and interferon-γ, and Th 2 cells, which secrete IL-4 and IL-5 (Mosmann *et al.*, 1986). Previous study by this group has shown that only Th 1 cells are involved in mediating DTH reaction (Cher and Mosmann, 1987). Based on these findings, the significant enhancement of DTH reaction and enlargement of the thymus in mice dosed at 63 mg/kg/day P-MA suggest that P-MA may enhance the cell-mediated immune responses to SRBC, at least in part, by enhancing either direct or indirect activation of mouse T cells which are associated with DTH reaction. Further studies are needed to investigate whether P-MA induces DTH reaction by certain other antigen.

Macrophages play a key role in antigen recognition and processing with subsequent interaction with T and B cells to initiate cell-mediated and humoral antibody responses (Feldmann, 1972; Tam and Hinsdill, 1984). Two components of macrophages must be considered. One is the fixed phagocytic cells in the reticuloendothelial system, the other is the mobile macrophages, represented by peritoneal exudate cells. They are known to release many cytokines that play important roles in maintaining homeostasis. Based on these findings, Table IV shows the effects of PM-A on phagocyte activity in ICR mice. Phagocyte activity was significantly increased in mice dosed at 63 mg/kg/day P-MA (*i.e.*, 4.99±0.27 at the dose of 63 mg/kg/day, p<0.05 as compared with 4.06±0.32 in controls).

The number of circulating leukocyte counts was also significantly increased in mice dosed at 63 mg/kg/day P-MA (*i.e.*, 7,399±446 at the dose of 63 mg/kg/day, p<0.05 as compared with 6,017±473 in con-

Table III. The effects of plantago-mucilage A on delayed-type hypersensitivity response in ICR mice

P-MA dose (p.o.) (mg/kg/day)	DTH reaction to SRBC ($\times 10^{-2}$ mm)
0	160±10
7	151± 9
21	175±12
63	192±10*[a]

Abbreviations: plantago-mucilage A, P-MA; delayed-type hypersensitivity, DTH. DTH reaction was determined by footpad swelling test. Footpad swelling is shown as the difference between the thickness of the footpads challenged with SRBC and saline, respectively. Each value represents the mean±S.E. of 10 mice. *a)* Asterisks denote a significant difference compared with the value in control mice that did not receive P-MA. *P<0.05

Table IV. The effects of plantago-mucilage A on phagocyte activity in ICR mice

P-MA dose (p.o.) (mg/kg/day)	Corrected phagocytic index[a]
0	4.06±0.32
7	4.10±0.19
21	4.73±0.37
63	4.99±0.27*[b]

Abbreviations: plantago-mucilage A, P-MA. *a)* Corrected phagocytic index is a constant obtained from a formula relating the cube root K to the ratio of body weight to the weights of the liver and spleen. Each value represents the mean±S.E. of 10 mice. *b)* Asterisks denote a significant difference compared with the value in control mice that did not receive P-MA. *P<0.05

Table V. The effects of plantago-mucilage A on the number of circulating leukocyte in ICR mice

P-MA dose (p.o.) (mg/kg/day)	Number of circulating leukocyte (/mm^3)
0	6,017±473
7	6,156±456
21	5,979±481
63	7,399±446*[a]

Abbreviations: plantago-mucilage A, P-MA. Blood samples for measuring leukocytes in mice were collected from the retro-orbital plexus immediately before assay. Each value represents the mean±S.E. of 10 mice. *a)* Asterisks denote a significant difference compared with the value in control mice that did not receive P-MA. *$P<0.05$

trols) (Table V). On the basis of the above findings, it was considered that an alteration in reticuloendothelial system function is a sensitive effect of oral administration to P-MA and seems to be primarily stimulative.

In conclusion, the results presented here demonstrate that P-MA significantly enhances immune functions according to the dose in ICR mice, without any alteration in the relative weight of liver which is the main storage organ of P-MA. However, further investigations are needed to elucidate the exact mechanisms which underlie these effects.

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