Protective Effect of Astragali Radix by Oral Administration against Japanese Encephalitis Virus Infection in Mice

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We investigated the protective effect of Astragali Radix (AR) by oral administration against Japanese encephalitis virus (JEV) infection in mice, the pharmacological effects of AR extracts (AE) in different origin, and the chemical composition of the AEs. A protective effect was demonstrated in all four AEs used, however, the effective grade for each one was different. In the control group, an increase of hemagglutination inhibition (HI) antibody titer was observed in all mice surviving 25 d after JEV inoculation. However, the increase of HI antibody titer was not observed in some animals administered an AE. In the control group, the rate of HI antibody positive mice was 90% 3 d after JEV inoculation, while the four groups which received the AE had a 30–60% positive rate. In mice which received the AE, the peritoneal exudate cell (PEC) numbers increased significantly compared to the control group. The predominant cell population of PECs in mice receiving the AE was macrophages, and in the PEC, the active oxygen (AO) production was high.

From these results, we propose that the protective effect of AE by oral administration is based on a non-specific mechanism during the early stage of infection, before shifting to antibody production, and that macrophages play an important role in this resistance to JEV infection, *e.g.*, by inducing the production of AO. In the chemical composition of each AE, carbohydrate was the major component.

Key words Astragali Radix; Japanese encephalitis virus; carbohydrate; oral administration; active oxygen; macrophage

Crude drugs are used as preventatives and treatment for various diseases, and they are nearly always administered orally. Astragali Radix (AR) is used in China to improve a naturally weak constitution and unbalanced nutrition. It is chiefly used as an decoction.

We previously reported the protective effect of Astragali Radix extracts (AE) by intraperitoneal (i.p.) injection against Japanese encephalitis virus (JEV) infection in mice, and the differences in the protective effect of AE samples.¹⁾

In the present study, we examined the protective effect of AE by oral administration against JEV infection in mice, the differences in the pharmacological effects of each AE sample, and the chemical composition of the AEs.

MATERIALS AND METHODS

Mice Male ICR mice were purchased from Charles River Japan (Yokohama, Japan). They were acclimatized to their housing for at least 7 d prior to the experiments. Five-week-old mice were used for all experiments.

Virus The virus was used at the 6th passage in suckling mouse (ddY strain) brain. The stock virus solution titer was determined by a plaque assay²⁾ using baby hamster kidney cells (1×10^7 PFU/ml).

Samples and Preparation of Extracts Four strains of AR (Locality: A, Shanshi; B, Hope; C, Hsiahs; D, Hokkaido) (Japanese locality names: A, Sansei-syo; B, Kahoku-syo; C, Sensei-syo; D, Hokkaido) were used as samples. All samples are the same as used in our previous report.¹⁾ AEs were prepared as described.¹⁾ Briefly, the samples (100 g) were added to distilled water (1000 ml), and extracted in a water bath at 80 °C for 2 h. After filtration, the extract solution was concentrated to one-fifth

its original quantity under reduced pressure at 40–50 °C, then added to methanol (2000 ml). After freeze-drying, the precipitate was used in experiments as the AE.

Method of AE Administration AE was dissolved in a phosphate buffer solution (PBS, 12 mg/ml), and orally administered to the mice (0.1 ml/mouse) by gastric tube once a day for 5 d. The control mice were administered PBS only.

Protective Effect of AE against JEV Infection The day after final AE oral administration, the mice received an i.p. inoculation of JEV (10 PFU) diluted with Tris-HCl solution (pH 7.4) containing 0.28% bovine serum albumin (BSA). The survival rates were observed for 25 d from this JEV inoculation. Twenty mice per group were used in this experiment.

Determination of Hemagglutination Inhibition (HI) Antibody Titer HI antibody titer was measured using a modification of Clarke and Casals' method.³⁾ In brief, HI antigen (purchased from Denkaseiken Inc., Tokyo, Japan) which was diluted by boric acid buffer (with 1% white egg albumin, pH 9.0) and serum treated with acetone were mixed and left standing overnight at 4 °C. The next day, red blood cells from 1-d-old chicks were added, and the antibody titer was measured with the inhibition of hemagglutination as an index. The HI antibody titer was expressed as the highest dilution of serum that caused inhibition of hemagglutination.

Effects of AE on Peritoneal Exudate Cell (PEC) Number The day after final AE administration, the PEC numbers in the mice were examined by the method of Robert.⁴⁾ In brief, after mice were killed by cutting the necks and resultant bloodletting, PBS (20 ml) cooled at 4 °C was impregnated into the peritoneum. The mouse abdomens were massaged and abdominal solution col-

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lected. The PEC number was calculated using a hemacytometer (Burker-Turk). Three mice per group were used in this experiment.

Examination of PEC Population The PEC population was determined by the May-Giemsa staining method.⁵⁾ In brief, PEC solution (2×10^6 cell/ml) prepared with bovine serum was smeared over a piece of slide. After being fixed with May-Grunwald solution (Kanto Chemical Co., Inc., Tokyo, Japan) for 2 min, the solution was stained with distilled water for 1 min, and then stained with Giemsa solution (Kanto) diluted with PBS ($\times 50$) for 20 min. After washing, the PEC population was examined by light microscope.

Effects of AE on Active Oxygen (AO) Production in PEC The productivity of AO in the PEC was measured as reported earlier.¹⁾ In brief, a pool of the collected PEC abdominal solution from three mice was used. Lucigenin (5 mM) was added (5 μ l per vial) to a cell solution (4×10^5 cells/ml) prepared with Roswell Park Memorial Institute medium with 3% BSA. After the solution was warmed at 20°C for 10 min, opsonized zymosan solution (10 mg/ml) was added (10 μ l per vial). Luminescence was measured using a liquid scintillation counter for 150 min. A parallel control experiment adding superoxide dismutase (SOD) was performed to confirm that the luminescence was caused by AO production.

Carbohydrate and Protein Assays Total carbohydrate was assayed by the phenol-sulfuric acid method,⁶⁾ using glucose as a standard. Protein was assayed by the Lowry method,⁷⁾ using BSA as a standard.

Statistical Analysis The survival percent data were analyzed by Cox-Mantel method, and the mean survival days and the PEC number data by one way analysis of variance. If there was significant difference among the groups, Fisher's PLSD test was conducted for multiple comparison. The rate of HI antibody positive mice data was analyzed by Fisher's exact probability test, and AO production data by Friedman test. If the *p*-value was below 0.05, we considered the result significant.

RESULTS

Protective Effects of AE against JEV Infection A decrease in the survival rate was observed from the 8th day after JEV inoculation (Fig. 1). The survival rate of the control group 25 d after JEV inoculation was 20%. In contrast, the groups administered an AE had 30–40% survival rates (Table 1). The mean number of survival days of mice administered an AE was greater than that of the control mice. Significant differences from the control group were found in all four AE-administered groups: A, $p < 0.001$; B, $p < 0.001$; C, $p < 0.01$; D, $p < 0.05$. Also, the mean number of survival days for each AE group was different. A significant difference was recognized between the groups administered sample A and D extracts ($p < 0.05$).

HI Antibody Titer in Mice Surviving 25 d after JEV Inoculation In the control group, increases of HI antibody titer were observed in all mice surviving 25 d after JEV inoculation (Fig. 2). In some mice administered each AE, however there was no increase of this titer. A

Fig. 1. Effects of AE on Survival Rate

Respective AEs were administered (1.2 mg/mouse) to mice once a day for 5 d; the next day, JEV was inoculated (10 PFU) (i.p.). 20 mice per group were used in this experiment. The control group was treated with PBS. ○, control group; ●, the group administered sample A (Sansei-syo) extract; □, sample B (Kahoku-syo) extract; △, sample C (Sensei-syo) extract; ■, sample D (Hokkaido) extract.

Table 1. Protective Effect of AE against JEV Infection

	No. of mice	MSD ^{a)} (d)	Survival rate (%) ^{b)}
Control	20	> 12.8	20
A***	20	> 16.4	35
B***	20	> 15.9	40
C**	20	> 15.2	30
D*	20	> 14.2	30

a) Mean survival days during the 25-d experimental period. b) Survival rate at 25 d after JEV inoculation. Control group was treated with PBS. A: The group administered sample A (Sansei-syo) extract, B: sample B (Kahoku-syo) extract, C: sample C (Sensei-syo) extract, D: sample D (Hokkaido) extract. Significantly different from control value at $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***).

Fig. 2. HI Antibody Titer in Mice Surviving 25 d after JEV Inoculation

The control group mice were treated with PBS. ○, control group; ●, the group administered sample A (Sansei-syo) extract; □, sample B (Kahoku-syo) extract; △, sample C (Sensei-syo) extract; ■, sample D (Hokkaido) extract. Significantly different from control value at $p < 0.05$ (*).

significant difference from the control group was revealed in the sample D extract-administered group ($p < 0.05$).

HI Antibody Titer in Mice on the 3rd and 7th Days after JEV Inoculation The HI antibody titers were examined on the 3rd and 7th days after JEV inoculation (Table 2). In the control group, the rate of HI antibody-positive mice

was 90% 3 d after JEV inoculation, while the groups which received an AE had 30–60% positive rates. Significant differences from the control group were recognized: sample

Table 2. Rate of HI Antibody-Positive Mice on the 3rd and 7th Days after JEV Inoculation

	No. of mice	Days after JEV inoculation	
		3th (%) ^{a)}	7th (%) ^{a)}
Control	10	90	90
A	10	40*	40*
B	10	40*	50
C	10	60	70
D	10	30**	60

a) Rate of antibody titer-positive mice. Each AE was administered once a day for 5 d (1.2 mg/mouse), and the next day, JEV was inoculated (10 PFU). Control group was treated with PBS. A: The group administered sample A (Sansei-syo) extract, B: sample B (Kahoku-syo) extract, C: Sample C (Sensei-syo) extract, D: sample D (Hokkaido) extract. Significantly different from control value at $p < 0.05$ (*), $p < 0.01$ (**).

Fig. 3. Effects of AE on PEC Number

Each column represents the mean ± S.E. of 3 mice. Respective AEs were administered (1.2 mg/mouse) to mice once a day for 5 d; the PEC number was measured the next day. The control group mice were treated with PBS. ○, control group; ●, the group administered sample A (Sansei-syo) extract; □, sample B (Kahoku-syo) extract; △, sample C (Sensei-syo) extract; ■, sample D (Hokkaido) extract. *, **, ***: Significantly different from control value, at $p < 0.05$, $p < 0.01$, $p < 0.001$.

Fig. 4 Effect of AE on AO Production

Respective AEs were administered (1.2 mg/mouse) to mice once a day for 5 d, and the next day, the productivity of AO in PEC obtained from mice was measured. ○, the cells obtained from mice treated with PBS; ●, the cells obtained from mice administered sample A (Sansei-syo) extract; □, sample B (Kahoku-syo) extract; △, sample C (Sensei-syo) extract; ■, sample D (Hokkaido) extract.

A, $p < 0.05$; B, $p < 0.05$; D, $p < 0.01$. On the 7th day, the rate of HI antibody titer-positive mice in the AE groups was also lower than that in the control group.

Effects of AE on PEC Number The PEC number data are shown in Fig. 3. Significant differences from the control group were seen for all four AE-administered groups (A, $p < 0.05$; B, $p < 0.01$; C, $p < 0.05$; D, $p < 0.001$). The difference in the PEC number between the mice administered the sample D extract and the control mice was remarkable. The predominant cell population of the PEC in the respective AE groups was macrophages (70–85%). The neutrophil, lymphocyte and mast cell populations were lower (data not shown). Differences in the cell populations among the AE-administered groups were negligible.

Effects of AE on AO Product in PEC The day after final AE administration, the productivity of AO in the PEC obtained from mice was measured. High AO productivity was observed in the PEC obtained from animals receiving an AE, compared to the control mice (Fig. 4). The differences in AO productivity between the PECs from mice administered each AE (the sample A, B and D extracts) and the control mice were remarkable. A significant difference among the respective AE groups was observed ($p < 0.001$). When the PEC from all groups were treated with SOD, the activity disappeared completely.

The Chemical Composition of AEs The chemical composition of each AE is shown in Table 3. Carbohydrate was the major component in each one (81.5–90.6%), and the protein component was lower (4.5–15.7%).

Table 3. Yields and Chemical Compositions of AE

Sample	Yield (%)	Carbohydrate (%) (as glucose)	Protein (%) (as BSA)
A	3.8	86.1	15.7
B	1.9	90.6	12.2
C	1.7	81.5	10.1
D	2.9	87.6	4.5

A: Sample A (Sansei-syo) extract, B: sample B (Kahoku-syo) extract, C: sample C (Sensei-syo) extract, D: sample D (Hokkaido) extract.

DISCUSSION

The protective effect of AE by oral administration against JEV infection in mice was demonstrated in the present study. HI antibody titers were one protective factor. In all of the control mice that survived 25 d after JEV inoculation, the HI antibody titer was increased. However, in some of those administered an AE, no titer increase was seen. This discrepancy was most notable in the early stage of JEV infection. In the control group at 3 d after JEV inoculation, the rate of HI antibody positive mice was 90%; the AE groups had 30–60% positive rates. JEV was reportedly observed in the blood of a mouse at 1 d after i.p. inoculation, and HI antibody titer in that mouse was increased at 3 d.⁸⁾ Since various primary factors are involved in protective mechanisms against virus infection, we speculated that the protective effect of AE might depend on a non-specific mechanism in the early stages of infection, before shifting to antibody production. There have been several observations of crude drugs affecting the accumulation and activity of PEC.⁹⁾ The ability of AR to enhance the phagocytosis of macrophages has been reported.¹⁰⁾ We surmised that PEC would play an important role in AE activity. The PEC numbers in the mice administered an AE were significantly higher than those of the control mice; the PEC obtained from the AE mice showed high AO productivity, and the predominant cell population of the PEC was macrophages.

From these results, we suggest that macrophages have an important function in protecting AE against JEV infection by oral administration.

We previously reported that the protective effect of AE by i.p. injection was dependent on a non-specific mechanism during the early stage of infection, before shifting to antibody production, and that PEC was a key player.¹⁾ The present study demonstrated the same action and mechanism of AE, when AE was administered orally. The effect of the drug Ninjin-yousei-to by i.p. injection against *Listeria monocytogene* infection has been reported to be based on the same mechanism during the early stage of infection.¹¹⁾ Irinoda *et al.* reported that lentinan produces a protective effect against airborne influenza virus infection through the stimulation of a non-specific mechanism.¹²⁾

From these studies and our results, we surmise that protection in the early stage is very important against viral or bacteria infections because they can grow exponentially with time.

We reported that the degree of protective effects against JEV infection differed among the AE injected (i.p.) groups.¹⁾ In the present study, the differences in the pharmacological effects of each AE sample were recognized. It is generally known that the quality of crude drugs differs from sample to sample. Many studies have been done on the biological effects of crude drugs, but very few have been conducted using the same kind of plural number samples. Such studies are necessary to see the exact effects of crude drugs.

We examined the relationship between the differences in AE effects and the chemical composition (protein and carbohydrate) of each AE, but observed no correlation between these factors. Further investigations will be necessary to elucidate the cause for differences in AE

effects.

In the chemical compositions of all four present AE samples, carbohydrate was found to be largest component. There have been several reports on the protective effects of glycoprotein and polysaccharide.¹³⁾ We suspected that the active ingredient in the AEs might also belong to that family of compounds. Quality evaluations of crude drugs are now accomplished by physical and chemical tests using low molecular weight compounds as indexes, or by the five senses method.¹⁴⁾ However, these tests cannot evaluate a drug's efficacy as medicine. Since herbal substances are used as medicine, new methods of quality evaluation which can determine the safety and effectiveness of herbal medicines must be established. It is widely noted that crude drugs can act on various diseases by enhancement of a protective power.¹⁵⁾ In the future, the development of quality evaluation methods for crude drugs will be important, perhaps using glycoprotein and polysaccharide as indexes.

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