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 AE. 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 ratio 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 adjuvant.

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 assay 2) using baby hamster kidney cells (1 × 10 5 PFU/ml).

Samples and Preparation of Extracts Four strains of AR (Locality: A, Shanshi; B, Hope; C, Hsiahsi; D, Hokkaido) (Japanese locality names: A, Sansi-syo; B, Kaho-su-so; 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 hemacy-
tometer (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. In
brief, PEC solution (2 × 10⁶ 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 (× 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. In brief, a pool of the collected
PEC abdominal solution from three mice was used.
Lucigenin (5 mM) was added (5 μl per vial) to a cell solution
(4 × 10⁶ 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 solu-
tion (10 mg/ml) was added (10 μl 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, using
glucose as a standard. Protein was assayed by the Lowry
method, 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
were 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 de-
crease 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](image1)

![Fig. 2. HI Antibody Titer in Mice Surviving 25 d after JEV Inoculation](image2)
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 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 2. Rate of HI Antibody-Positive Mice on the 3rd and 7th Days after JEV Inoculation

<table>
<thead>
<tr>
<th>Sample</th>
<th>No. of mice</th>
<th>3rd (%)*</th>
<th>7th (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10</td>
<td>90</td>
<td>90</td>
</tr>
<tr>
<td>A</td>
<td>10</td>
<td>40*</td>
<td>40*</td>
</tr>
<tr>
<td>B</td>
<td>10</td>
<td>40*</td>
<td>50</td>
</tr>
<tr>
<td>C</td>
<td>10</td>
<td>60</td>
<td>70</td>
</tr>
<tr>
<td>D</td>
<td>10</td>
<td>30**</td>
<td>60</td>
</tr>
</tbody>
</table>

*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 (Salsei-syo) extract; **B**: sample B (Kahoku-syo) extract; **C**: Sample C (Salsei-syo) extract; **D**: sample D (Hokkaido) extract. Significantly different from control value at p < 0.05 (*), p < 0.01 (**).

### Table 3. Yields and Chemical Compositions of AE

<table>
<thead>
<tr>
<th>Sample</th>
<th>Yield (%)</th>
<th>Carbohydrate (%) (as glucose)</th>
<th>Protein (%) (as BSA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>3.8</td>
<td>86.1</td>
<td>15.7</td>
</tr>
<tr>
<td>B</td>
<td>1.9</td>
<td>90.6</td>
<td>12.2</td>
</tr>
<tr>
<td>C</td>
<td>1.7</td>
<td>81.5</td>
<td>10.1</td>
</tr>
<tr>
<td>D</td>
<td>2.9</td>
<td>87.6</td>
<td>4.5</td>
</tr>
</tbody>
</table>

A: Sample A (Salsei-syo) extract; B: sample B (Kahoku-syo) extract; C: sample C (Salsei-syo) extract; D: sample D (Hokkaido) extract.

![Fig. 3. Effects of AE on PEC Number](image)

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 (Salsei-syo) extract; ■, sample B (Kahoku-syo) extract; △, sample C (Salsei-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](image)

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 (Salsei-syo) extract; △, sample B (Kahoku-syo) extract; △, sample C (Salsei-syo) extract; ■, 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 25d 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 3d 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 1d after i.p. inoculation, and HI antibody titer in that mouse was increased at 3d.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.11 The present study demonstrated the same action and mechanism of AE, when AE was administered orally. The effect of the drug Ninjin-youei-to by i.p. injection against Listeria monocytogenes infection has been reported to be based on the same mechanism during the early stage of infection.11 Irinoda et al. reported that lentinian 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.11 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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REFERENCES