Protective Effect of Astragalus Radix by Intraperitoneal Injection against Japanese Encephalitis Virus Infection in Mice

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We examined the protective effect of Astragalus Radix extracts (AE) by intraperitoneal injection against Japanese encephalitis virus (JEV) infection in mice. A protective effect was observed by all four samples of AE used. However, the degree of effectiveness for each AE was different. The observed survival rates of the groups injected with sample A (from Shanhs, Japanese name Sansei-syo) and sample D (from Hokkaido) extracts were higher than 80% at 21 d after JEV inoculation. The groups injected with sample B (from Hopei, Japanese name Kahoku-syo) and sample C (from Hsiabs, Japanese name Semei-syo) extracts had a 60% survival rate. The increase in hemagglutination inhibition antibody titer was negligible in mice that survived 21 d after JEV inoculation. The antiviral effect of AE was examined by plaque assay in vitro, but no antiviral effect was shown. In mice injected with AE, the peritoneal exudate cell (PEC) numbers increased significantly, compared to the control. In these PEC, active oxygen production was also high. Also the group as a whole displayed a high survival rate against JEV infection, these were so strong.

From these results, we propose that the protective effect of AE is dependent on a non-specific mechanism during the early stage of infection, before it shifts to antibody production, and that PEC plays an important role.

Key words Astragalus Radix; Japanese encephalitis virus; peritoneal exudate cell; active oxygen; hemagglutination inhibition antibody titer

Astragalus Radix (AR) is an important traditional Chinese herbal medicine widely used in China and Japan. It is used to improve naturally weak constitutions and to counter unbalanced nutrition in China. Chinese herbal medicines are used as preventative and the treatment for various viral infections, and they apparently enhance the immunological powers of the host. It has been reported that the Chinese herbal medicine Shosaiko-to can act against hepatitis virus infection by augmenting the host's resistance, e.g., by inducing the production of interferon.1 Ikemoto et al. reported that Shigaku-to produces a protective effect against the herpes simplex virus through the activation of CD8+ T cells.2 Various effects of Ginseng Radix on the immune system have also been reported,3 such as its protective effect against Japanese encephalitis virus (JEV) infection.4 There have been many reports on the protective effects of AR, including its enhancement of antibody production,5 acceleration of macrophage phagocytosis,6 increase of tumor necrosis factor production,7 activity on the reticuloendothelial system,8 and its enhancement of natural killer cell activity.9 Thus, the protective effects of AR against viral infections could be expected. However, no detailed study of the effect of AR against viral infections has been reported.

It is generally known that crude drug quality differs from batch to batch. Even though AR is not an exception, it has been reported that the contents of isoflavonoids, canavanine, γ-amino butyric acid and astragaloside, which are indexes of chemical quality control, differ from sample to sample.10 Also, extreme differences in the outer appearance of AR occur in the market.11 Many studies have been done on the biological effects of crude drugs, but very few studies have investigated differences in the biological effect of the same drug.

In this study, we examined the protective effect of Astragalus Radix extracts (AE) against JEV infection, and the differences in the biological effects of each AE sample.

MATERIALS AND METHODS

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

Viruses JEV (JaTH-160 strain) was kindly provided by Dr. Ueba (Osaka Prefectural Institute of Public Health, Osaka, Japan). 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 using Baby Hamster Kidney (BHK-21) cells (1 × 10⁵ PFU per plaque forming unit/ml).

Samples and Preparation of Extracts Four strains of AR (Locality: A, Shanhs; B, Hope; C, Hsiabs; D, Hokkaido) (Japanese names by locality: A, Sansei-syo; B, Kahoku-syo; C, Sensei-syo; D, Hokkaido) were used as samples. All of the samples were standardized articles of the Japanese Pharmacopoeia.12 Extracts of the samples were prepared according to the method of Yamasaki et al.13 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 AE.

Protective Effect of AE against JEV Infection in Vivo

Toxicity testing was carried out using three cell lines (BHK-21, L-929, Vero).14 The lowest concentration non-toxic to the cell was chosen as the dose. AE was dissolved in a phosphate buffer solution (PBS, 30 mg/
ml), and injected intraperitoneally (i.p.) into the mice (0.2 ml/mouse). The control mice were injected with PBS only. The next day, the mice received an i.p. inoculation of JEV (10 PFU) diluted by Tris-HCl solution (pH 7.4) containing 0.28% bovine serum albumin (BSA). The survival rates were observed for 21 d from the JEV inoculation. Twenty mice per group were used in this experiment.

**Antiviral Effect of AE in Vitro** Plaque assays were performed using a slightly modified version of the Kimura et al. method. JEV diluted by the buffer described above (1 x 10^6 PFU/ml), and AE (30 mg/ml) diluted by PBS, were mixed and left standing for 1 h at 37°C. The mixture was then inoculated into BHK-21 cells. After these were cultured in Eagle's basal supplemented medium with 7% calf serum for 48 h at 37°C (5% CO2), the plaque that formed on the BHK-21 cells was measured and the plaque number was recorded as an index of antiviral effect.

**Determination of Hemagglutination Inhibition (HI) Antibody Titer** HI antibody titer was measured using a modification of Clarke and Casals' method. In brief, HI antigen (purchased from Denkaseiken, Inc., Japan), which was diluted by boric acid buffer (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 one-day 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 the inhibition of hemagglutination.

**Effects of AE on Peritoneal Exudate Cell (PEC) Number** PEC numbers were calculated in the mice from the first to the 5th day after AE injection (6 mg/mouse). Calculation of the PEC number was performed by the method of Robert. After mice were killed by cutting the necks and after the resultant bloodletting, PBS (20 ml) cooled at 4°C was impregnated into the peritoneal. After the abdomens of the mice were massaged, abdominal solution was collected. Calculation of the PEC number was performed using a hemacytometer (Burker-Turk). Three mice per group were used in this experiment.

**Effects of AE on Active Oxygen (AO) Production in PEC** The productivity of AO in the PEC obtained from mice 1 d after AE injection (6 mg/mouse) was measured by luminol-dependent chemical luminescence. A pool of the collected PEC abdominal solution from three mice was used in this experiment. Lucigenin (5 μM) was added (5 μl per vial) to the cell solution (4 x 10^6 cells/ml) prepared by Roswell Park Memorial Institute medium with 3% BSA. After this was kept warm at 20°C for 10 min, opsonized zymosan solution (10 mg/ml) was added (10 μl per vial). Luminescence was measured using a liquid scintillation counter for 140 min. We ran a parallel control experiment adding SOD (superoxide dismutase) to confirm that the luminescence was caused by AO production.

**Statistical Analysis** All data were analyzed as follows: survival percent by logrank analysis; PEC number and plaque number by Student’s t-test. If the p-value was below 0.05, we considered the results significant.

**RESULTS**

**Protective Effects of AE against JEV Infection in Vivo** In the control group, a sharp decrease in the survival rate was observed from the 7th day to the 16th day after JEV inoculation. In contrast, in none of the groups injected with AE was a similar decrease in survival rate observed. The protective effects against JEV infection were demonstrated in all four experimentally used AE (Fig. 1). Significant differences from the control group were found in the respective AE-injected groups: A, p < 0.01; B, p < 0.05; C, p < 0.05; D, p < 0.01. However, the survival rate at 21 d after virus inoculation was different for each AE-injected group. The survival rates of the groups injected with sample A or D extracts were higher than 80%, but the groups injected with sample B or C extracts had 60% survival rates. A significant difference was recognized between the groups injected with C and D extracts (p < 0.05).

**Effects of AE on Body Weight** The long-term changes in body weight (mean weight of surviving mice) are shown in Fig. 2. In the control group, a sharp decrease in body weight was seen from the 7th day to the 12th day after JEV inoculation, but in the groups injected with AE, there were only slightly scattered, slight body weight decreases. The groups injected with samples A and D extracts, which had survival rates higher than 80%, showed decreases in weight the day after AE injection. In contrast, weight decreases were rarely exhibited in the groups injected with samples B and C extracts, where the survival rates were approximately 60%. On the other hand, in the control group, body weight increases were recognized.

**HI Antibody Titer in Mice** The HI antibody titer was measured in mice that survived 21 d after virus inoculation (Fig. 3). Antibody titer increases were recognized only in two mice that had been injected with sample B extract.

**Antiviral Effect of AE in Vitro** We examined the antiviral effect of AE in vitro (Table 1). No significant dif-

**Fig. 1. Effects of AE on Survival Rate in Mice**

Respective AE were injected (6 mg/mouse) in mice 1 d before JEV inoculation (10 PFU). 20 mice per group were used in this experiment. The control group was treated with saline. (), control group; (), the group injected with sample A (Susen-kyo) extract; ( ), the group injected with sample B (Kahoku-kyo) extract; ( ), the group injected with sample C (Senso-jyo) extract; ( ), the group injected with sample D (Hokkaido) extract.

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Fig. 2. Body Weight Changes in Mice
The respective AE were injected (6 mg/mouse) in mice 1 d before JEV inoculation (10 PFU). The control group was treated with saline. Each point is the average weight of survival mice. ©, control group; ●, the group injected with sample A (Sansen-syo) extract; □, the group injected with sample B (Kahoku-syo) extract; △, the group injected with sample C (Sensei-syo) extract; ■, the group injected with sample D (Hokkaido) extract.

Fig. 3. HI Antibody Titer in Mice that Survived 21 d after JEV Inoculation
Control group mice were treated with saline. A, the group injected with sample A (Sansen-syo) extract; B, the group injected with sample B (Kahoku-syo) extract; C, the group injected with sample C (Sensei-syo) extract; D, the group injected with sample D (Hokkaido) extract.

Fig. 4. Effects of AE on PEC Number in Mice
Each point represents the mean ± S.E. of 3 mice. The respective AE were injected (6 mg/mouse). The control group was treated with saline. ©, control group; ●, the group injected with sample A (Sansen-syo) extract; □, the group injected with sample B (Kahoku-syo) extract; △, the group injected with sample C (Sensei-syo) extract; ■, the group injected with sample D (Hokkaido) extract. **, significantly different from control value at p < 0.01; *, significantly different from control value at p < 0.05.

Table 1. Antiviral Effect of AE in Vitro

<table>
<thead>
<tr>
<th>Sample</th>
<th>PFU (10⁶/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.63 ± 0.34</td>
</tr>
<tr>
<td>A</td>
<td>1.95 ± 0.44</td>
</tr>
<tr>
<td>B</td>
<td>3.58 ± 0.70</td>
</tr>
<tr>
<td>C</td>
<td>3.45 ± 0.65</td>
</tr>
<tr>
<td>D</td>
<td>2.08 ± 0.41</td>
</tr>
</tbody>
</table>

Each value represents the mean ± S.E. of 4 dishes. Significant differences from the control were not recognized. Control, saline; A, sample A (Sansen-syo) extract; B, sample B (Kahoku-syo) extract; C, sample C (Sensei-syo) extract; D, sample D (Hokkaido) extract.

Effects of AE on PEC Number
The PEC numbers were calculated from the first to the 5th day after AE injection (Fig. 4). The PEC numbers of mice injected with AE reached their maximum values on the second day. Significant differences from the control group were recognized in each AE-injected group, and the differences on the second day were the largest. The PEC numbers of the mice injected with samples A and D extracts were higher than those of the mice injected with samples B and C extracts. For the cell population of PEC in the mice injected with AE, the neutrophil ratio was the highest on the next day and the macrophage ratio was the highest on the 5th day after the injection (cell population was examined using the May-Gimzta staining method). [8]

DISCUSSION
Our results demonstrated the protective effect of AE by i.p. injection against JEV infection in mice. As one measure of the protective factor, HI antibody titers were examined in mice that survived 21 d after JEV inoculation. Except for in two mice, antibody titer increases were not observed. Various primary factors are involved in protective mechanisms against virus infection. From our results, it appears that the protective effect of AE depended on a non-specific mechanism in the early stages of
infection, before a shift to antibody production. Though the antiviral effect of AE was examined in vitro, we did not discern this effect.

There have been several observations of crude drugs affecting the accumulation and activity of PEC.\textsuperscript{19, 20} The ability of AR to enhance the phagocytosis of macrophages has also been reported.\textsuperscript{21} The effect of the drug Ninjin-youei-to by i.p. injection against \textit{Listeria monocytogenes} infection has been reported to be based on the enhanced accumulation and activity of peritoneal macrophages.\textsuperscript{20}

From these study results, we surmised that PEC would play an important role in AR activity, and we examined the effects of AE on PEC number and AO production. The PEC numbers in mice that were injected with AE were significantly higher than those of the control mice, and the ability of the PEC obtained from mice that were injected with AE showed high AO productivity. From these results, it appears that PEC plays an important role in AE protection against JEV infection.

Protective effects against JEV infection were observed in all of the AE, but the degree of effectiveness differed among the AE-injected groups. Biological effect differences for each AE were recognized. There have been many reports that the physical and chemical quality of a crude drug may differ from sample to sample, but very little has been reported about differences in the biological quality of the same drug; this paper is the first to do so regarding AR. Further studies are necessary to determine the exact cause of the differences among the samples; e.g., whether it is because of differences in the original plants or due to batch-to-batch differences. \textit{Astragalus membranaceus} \textit{Bunge} and \textit{Astragalus mongholicus} \textit{Bunge} are known as AR original plants in the Japanese Pharmacopoeia.\textsuperscript{12}

In our study, a decrease in body weight was observed from the time of injection to day one in the mice who received AE (samples A, D). In those mice, the PEC number increased greatly, and AO production was high in the PEC. It is known that active oxygen effects damage, not only in bacteria and viruses, but also in the living body.\textsuperscript{21} We suspect, therefore that the production of AO would be one of the primary factors in producing body weight decreases. In those AE, the protective effect against JEV infection \textit{in vivo} was great. In order to prevent toxicity, further investigation will be necessary.

At present, quality evaluation of crude drugs is accomplished by physical and chemical tests or by the five senses method. However, these cannot evaluate a drug's efficacy as medicine. Since herbal substances are used as medicine, new methods of quality evaluation that can determine the safety and effectiveness of herbal medicines must be established. The development of biological quality evaluation methods for crude drugs will be important in the future.

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REFERENCES AND NOTES

14) Data was not published.