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Protective effect of Shigyaku-to, a traditional Chinese herbal medicine, on the infection of herpes simplex virus type 1 (HSV-1) in mice

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Abstract. The antiviral activity of Shigyaku-to (TJS-109), a traditional Chinese herbal medicine, was investigated in mice infected with herpes simplex virus type 1 (HSV-1). TJS-109 is a combination of the medicinal plant extracts from *Zingiberis siccatum* rhizoma, *Aconiti* tuber and *Glycyrrhizae* radix in a specific proportion. Mice infected with a 10 LD_{50} dose of HSV-1 were treated with TJS-109 orally at doses of 1.25 to 20 mg/kg 2 days before, and 1 and 4 days after the infection. The treated groups had 80% (1.25 mg/kg), 40% (5 mg/kg) and 23% (20 mg/kg) mortality rates 25 days after the infection as compared with a 100% mortality rate in control mice treated with saline. When HSV-1 infected mice (recipients) received $CD8^+$ T cell fractions derived from spleens of mice treated with TJS-109 (donors), 70% of recipients survived, as compared with 0% survivors in the groups of mice treated with saline, B cell fractions, $CD4^+$ T cell fractions or macrophage-enriched fractions prepared from the same donors. TJS-109 did not show any virucidal activities against HSV-1 or any virostatic activities on the growth of HSV-1 in Vero cells. These results suggest that TJS-109 protected mice exposed to lethal amounts of HSV-1 through the activation of $CD8^+$ T cells.
Key words. Traditional Chinese herbal medicine; herpes simplex virus; antiviral effects; $CD8^+$ T cells.

Traditional Chinese herbal medicines are a combination of the crude extracts from several herbs and medicinal plants in a specific proportion. The prescription of one of the extracts alone is not common. The various extracts are said to exert a synergistic effect. Traditional Chinese herbal medicines have been used in China and Japan for hundreds of years. Recently, traditional Chinese herbal medicines have become increasingly popular as treatments in Japan, because some of them have been accepted as doctors' prescriptions in Japanese hospitals. More than 120 prescriptions in these medicines are currently available to treat cancer[1], allergic diseases[2], bacterial infections[3], viral infections[4] and other ailments, despite there being no strict scientific basis for their use. They are being used clinically on the basis of traditional experience.

Shigyaku-to (TJS-109), one of these traditional Chinese herbal medicines, is a combination of the extracts from 3 medicinal plants including *Glycyrrhizae* radix, *Zingiberis siccatum* rhizoma and *Aconiti* tuber at a ratio of 3:2:1[5]. This agent has also been used traditionally for treatment of various infections for thousands of years in China[5]. In this report, we investigated the antiviral activity of TJS-109 in mice and in tissue culture cells infected with herpes simplex virus type 1 (HSV-1). Results obtained suggest that TJS-109 protects mice against lethal amounts of HSV-1 through the activation of $CD8^+$ T cells.

Materials and methods

Mice, cells and viruses. Seven- to 8-week-old male BALB/c mice, obtained from The Jackson Laboratories (Bar Harbor, Maine, USA), were used in experiments. Vero cells were grown in Eagle's modified minimum essential medium (EMEM) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. The KOS strain of HSV-1 was propagated in Vero cells and stored at −70 °C until use[6]. The titer (determined by a plaque assay using Vero cells) of the stock virus solution was 1.8×10^7 PFU/ml[6]. In our experimental system, 4×10^3 PFU/kg was equivalent to a 1 LD_{50} dose in BALB/c mice inoculated intraperitoneally (i.p.)[6].

Reagents. TJS-109 was supplied from Tsumura & Co., Ltd. (Tokyo, Japan). With sonication, TJS-109 was dissolved in saline at the appropriate concentration. In this study it was administered orally into mice infected with HSV-1. Anti-L3T4 and anti-Lyt 2.2 monoclonal antibodies (mAb) were purchased from Accurate Chemical and Scientific Corporation (Westbury, New York, USA), and the low toxic M rabbit complement was obtained from Cedarlane Laboratories (Hornby, Ontario, Canada).

In vivo antiviral test. BALB/c mice, infected i.p.with a 10 LD_{50} dose of HSV-1, were treated orally with 0.2 ml/mouse of appropriate concentrations of TJS-109. Mice were treated with various doses (1.25–80 mg/kg) of the

agent 2 days before, and 1 and 4 days after the infection. Infected mice treated with saline (0.2 ml/mouse) served as controls. The antiviral activity of the agent was evaluated 25 days after the viral infection based upon the mortality rate and mean survival time in days (MSD) of the infected mice[6]. The number of survivors or MSD in the drug-treated groups was compared with that of control groups by means of χ^2 analysis (survival %) or Student's t-test (MSD)[6]. If the p value obtained was below 0.05, the drug dose was considered to have antiviral activity[6].

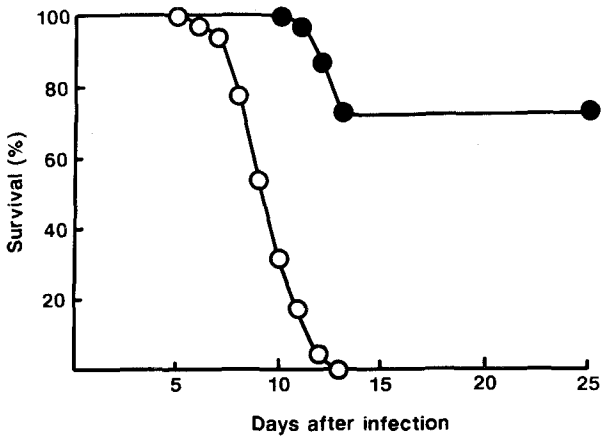
In vitro antiviral test. To determine antiviral activities of TJS-109 in tissue culture cells, its cytotoxic activities were determined first. Cytotoxicity was determined by the cytopathic effect and growth inhibitory effect of the agent on the monolayer cultures of Vero cells[7]. Using non-cytotoxic concentrations, the antiviral activity of TJS-109 was determined in Vero cells infected with HSV-1[8]. Vero cells cultured in 24-well plates were preincubated with 30 to 500 μg/ml of TJS-109 for 2 h at 37 °C. After adsorption of 5–100 PFU/ml of HSV-1 for 1 h, these cells were treated again with 30–500 μg/ml of TJS-109 and incubated for 24 h, 48 h or 72 h at 37 °C. Cells were then frozen and thawed three times. After centrifugation (1200 × *g*, 30 min), the supernatants of these cell cultures were tested for their viral titers in Vero cells by our standard plaque titration methods[6].

Splenic effector cells. As described previously[9], spleens were obtained aseptically from mice treated with or without the agent, and single cell suspensions (whole spleen cells, WSC) were prepared from these spleens. In some experiments WSC were separated into two fractions: plastic surface adherent cells (PAC) and plastic surface non-adherent cells (non-PAC)[9]. Briefly, WSC (2 to 5×10^7 cells/10 ml) were placed into petri dishes coated with FCS. The dishes were then incubated for 15 min at 37 °C to allow macrophages to attach. At the end of the incubation period, floating cells were removed with warm RPMI 1640 medium supplemented with 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer. PAC were harvested by scraping them from the dishes with a rubber scraper, after the dish was kept for 10 min at 4 °C. For the preparation of non-PAC, WSC suspended in the media at 2×10^7 cells/10 ml were introduced to FCS-coated petri dishes. The dishes were kept for 60 min at 37 °C to allow adherent cells to attach, non-PAC were harvested by gently swirling the dishes. Two additional 60-min incubations of the recovered non-PAC were performed on the FCS-coated petri dishes. The non-PAC obtained by this procedure contained 1% or fewer phagocytic macrophages[9]. To obtain T or B cell fractions, the non-PAC fraction was further fractionated by a nylon wool column[10]. Cells passed through a nylon wool column were used as enriched T cells (WTC). Cells which adhered to the nylon wool were subsequently recovered by compression of the nylon wool with the plunger of the syringe and used as a B cell-enriched fraction. WTC were further separated into $CD4^+$ T cells and $CD8^+$ T cells[11]. In order to prepare the $CD8^+$ T cell fraction, WTC were treated with a 1:200 dilution of anti-L3T4 mAb followed by complement for 30 min at 37 °C. In order to prepare the $CD4^+$ T cell fraction, WTC were treated with a 1:50 dilution of anti-Lyt 2.2 mAb and complement for 30 min at 37 °C. After treatment of the cell fraction with mAbs, the remaining cells were washed with media twice and resuspended in serum-free media at appropriate concentrations. Treatment of WTC, $CD4^+$ T cell fractions and $CD8^+$ T cell fractions with anti-CD3ε mAb plus complement resulted in a 99% reduction of viable cells. Treatment of $CD4^+$ or $CD8^+$ T cell fractions with anti-L3T4 mAb plus complement resulted in a 99% or 1% reduction of viable cells, respectively. In contrast, treatment of $CD4^+$ or $CD8^+$ T cell fractions with anti-Lyt 2.2 mAb plus complement resulted in a 1% or 99% reduction of numbers of viable cells. These results indicate that the purity of these cell preparations is more than 99%.

Adoptive transfer of various preparations of splenic cells to mice exposed to HSV-1. To examine in vivo antiviral mechanisms of TJS-109, mice exposed to lethal amounts of HSV-1 were treated with various splenic effector cells from mice injected with TJS-109. Mortality rates and mean survival time in days (MSD) of these mice were compared with those of control mice treated with saline. The following cells were adoptively transferred i.v. to the mice: WSC (whole spleen cells, 2×10^7 cells/mouse), PAC (macrophage-enriched fraction, 5×10^6 cells/mouse), non-PAC (macrophage-free splenic lymphocyte fraction, 1×10^7 cells/mouse), WTC (T cell-enriched fraction derived from non-PAC, 1×10^7 cells/mouse), B cell-enriched fraction derived from non-PAC (1×10^7 cells/mouse), WTC depleted with $CD4^+$ T cells ($CD8^+$ T cell fraction, 5×10^6 cells/mouse) and WTC depleted with $CD8^+$ T cells ($CD4^+$ T cell fraction, 5×10^6 cells/mouse). Two hours after the adoptive transfer, recipient mice were exposed to a 10 LD_{50} dose of HSV i.p., and survival % and the MSD of these mice were compared with those of controls treated with saline.

Results

As shown in the figure, when 30 infected mice were treated orally with a 20 mg/kg dose of TJS-109, 74% of them survived more than 25 days after the infection, while all controls (30 mice) treated with saline died within 13 days. The dose-response antiviral effect of TJS-109 on the HSV-1 infection in mice is shown in table 1. The highest protective effect was revealed when infected mice were treated with TJS-109 at a dose of 20 mg/kg (77% survival in treated mice vs 0% survival in the control, $p < 0.001$; >21.3 MSD in treated mice vs 9.6 MSD in the control, $p < 0.001$). As compared with



Effect of TJS-109 on the HSV-1 infection in mice. 30 mice infected with a 10 LD_{50} dose of HSV-1 were treated orally with a 20 mg/kg dose of TJS-109 2 days before, and 1 and 4 days after the infection (●). As a control (○), 30 mice treated with saline were infected with the same amount of HSV-1.

Table 1. Effect of various doses of TJS-109 on the survival of mice infected with HSV-1.

TJS-109[a] (mg/kg)	No. of mice	Survival (%)[b]	Mean survival time (days)[b]
0 (Saline)	30	0 (0)	9.5
1.25	10	2 (20)[c]	>14.2[d1]
5	10	6 (60)[c]	>18.8[d2]
20	30	23 (77)[c]	>21.3[d2]
80	10	0 (0)	8.5

[a]Mice infected with a 10 LD_{50} dose of HSV-1 were treated orally with various doses TJS-109 2 days before, and 1 and 4 days after the infection.
[b]The protective effect of TJS-109 was evaluated 25 days after the viral infection based upon the mortality (survival %) and mean survival time in days of the treated groups as compared with those of control groups treated with saline.
[c]χ^2 analysis, $p < 0.001$.
[d]Student's t-test, [d1]$p < 0.02$, [d2]$p < 0.001$.

the control (0% survival), significant reduction in mortality of infected mice was demonstrated when they were treated with TJS-109 at doses of 1.25 mg/kg (20% survival, $p < 0.001$; >14.2 MSD, $p < 0.02$) and 5 mg/kg (60% survival, $p < 0.001$; >18.8 MSD, $p < 0.001$). No protective effect was observed in mice treated with TJS-109 at a dose of 80 mg/kg. All of the infected mice treated with this amount of TJS-109 died within 9 days (8.5 MSD), which was a shorter period than for controls (9.6 MSD), suggesting that the administration of TJS-109 at a dose of 80 mg/kg induced some toxic side effects in mice. These results suggested that TJS-109 have clear antiviral activities in mice exposed to a lethal amount of HSV-1. Therefore, in the next experiment, possible antiviral mechanisms of the agent were studied. The viability of HSV-1 (10^5 PFU/ml) was not reduced by incubation with a 250 µg/ml concentration of TJS-109 in EMEM at 37 °C for 1 h. The cytotoxic effect of TJS-109 at concentrations of more than 500 µg/ml was demonstrated in both CPE tests and growth inhibition tests using Vero cells (data not shown). Concentrations less than 250 µg/ml of TJS-109 did not show any cytotoxicity in Vero cell cultures or any inhibitory activities on the growth of HSV-1 in these cells (data now shown). Since TJS-109 possessed neither virucidal nor virostatic activities in vitro, it was suggested that the agent expressed its antiviral effect through host functions in HSV-1 infected mice. Therefore, instead of the TJS-109 administration, various effector cells derived from TJS-109-treated mice were adoptively transferred to mice exposed to a 10 LD_{50} dose of HSV-1. As shown in table 2, infected mice (recipients) were inoculated with whole spleen cells (WSC) obtained from uninfected mice (donors) treated with TJS-109. The survival of recipients was increased to 60% as compared with 0% survival of control mice ($p < 0.001$). However, the adoptive transfer of

Table 2. Effect of adoptive transfer of various splenic cell fractions on the mortality and mean survival time of mice exposed to a 10 LD_{50} dose of HSV-1.

Infected mice were treated with[a]:	No. of mice	Survival (%)[b]	Mean survival time (days)[b]
Saline	30	0 (0)	9.8
Whole spleen cells (WSC)	10	6 (60)[c]	>19.4[d]
PAC prepared from WSC	10	0 (0)	9.0
Non-PAC prepared from WSC	10	6 (60)[c]	>20.0[d]
B cells prepared from non-PAC	10	0 (0)	9.8
T cells prepared from non-PAC	18	12 (67)[c]	>20.3[d]

[a]Splenic cells from mice treated with TJS-109 (20 mg/kg, oral) were fractionated into WSC (whole spleen cells), macrophage-enriched fractions (PAC, plastic adherent cells), macrophage-depleted fractions (non-PAC, plastic non-adherent cells), B cell-enriched fractions (nylon wool adhered non-PAC) or whole T cells fractions (nylon wool non-adhered non-PAC). Two hours before the infection of HSV-1 at a dose of 10 LD_{50}, these cell preparations were adoptively transferred i.v. to mice at cell numbers of 2×10^7 cells/mouse (WSC), 5×10^6 cells/mouse (PAC), 1×10^7 cells/mouse (non-PAC), 1×10^7 cells/mouse (WTC) and 1×10^7 cells/mouse (B cells).
[b]The protective effect of transferred cells was evaluated 25 days after the viral infection based upon mortality (survival %) and survival time (mean survival time in days) of the treated groups as compared with those of control groups treated with saline.
[c]χ^2 analysis, $p < 0.001$.
[d]Student's t-test, $p < 0.001$.

Table 3. Effect of adoptive transfer of T cell subsets, derived from spleens of TJS-109-treated mice, on the mortality and mean survival time of mice infected with a 10 LD_{50} dose of HSV-1.

Recipients were treated with[a]:	No. of mice	Survival (%)[b]	Mean survival time (days)[b]
Saline	30	0 (0)	10.1
T cells prepared from non-PAC (WTC)	18	12 (67)[c]	>20.3[d]
$CD4^+$ T cell-depleted WTC	10	7 (70)[c]	>22.0[d]
$CD8^+$ T cell-depleted WTC	10	0 (0)	9.8

[a]Mice exposed to a 10 LD_{50} dose of HSV-1 were treated i.v. with 1×10^7 cells/mouse of WTC (whole T cell populations), 5×10^6 cells/mouse of $CD4^+$ T cells ($CD8^+$ T cell-depleted WTC) or 5×10^6 cells/mouse of $CD8^+$ T cells ($CD4^+$ T cell-depleted WTC), derived from WSC of TJS-109-treated mice.
[b]The protective effect of transferred cells was evaluated 25 days after the viral infection based upon the mortality (survival %) and survival time (mean survival time in days) of treated groups as compared with those of the control group treated with saline.
[c]χ^2 analysis, $p < 0.001$.
[d]Student's t-test, $p < 0.001$.

macrophage-enriched fractions (PAC) or B cell enriched fractions prepared from these WSC resulted in no change in the mortality and morbidity of recipient mice exposed to the virus. These results indicated that the protective effect of TJS-109 in mice infected with HSV-1 might be expressed through the function of lymphocyte populations contained in spleens of TJS-109-treated mice. In fact, PAC-depleted WSC (non-PAC) protected mice from death due to HSV-1 (60% survival, $p < 0.001$). The protective activity of effector cells prepared from TJS-109-treated mice was also displayed when recipients were inoculated with whole T cell fractions prepared from non-PAC populations (WTC, 67% survival, $p < 0.001$). WTC fractions were further fractionated into $CD4^+$ T cell-depleted WTC and $CD8^+$ T cell-depleted WTC to examine what subpopulation of T cells was responsible for the antiviral activity of TJS-109 (table 3). Recipients treated with $CD4^+$ T cell-depleted WTC had a 70% survival rate ($p < 0.001$) and a MSD > 22 ($p < 0.001$), while the group treated with $CD8^+$ T cell-depleted WTC had a 0% survival rate and a 9.8 MSD. At this time, a 0% survival rate and a 10.1 MSD was demonstrated in the control group treated with saline. These results suggest that TJS-109 might protect mice from the infection of HSV-1 through its immunostimulating activity mediated by $CD8^+$ T cells.

Discussion

In this study we demonstrated the antiviral activity of TJS-109 in mice which were infected with a lethal dose of HSV-1. As compared with controls, the survival rate and mean survival time in days (MSD) of mice exposed to HSV-1 were significantly increased when they were treated orally with TJS-109 at doses ranging from 1.25 mg/kg–20 mg/kg. The 20 mg/kg oral dose of TJS-109 was found to be the most effective of the doses tested with infected mice, having significant improvements in survival rates. Since the agent did not show any virucidal or virostatic activities against HSV-1, it has been suggested that TJS-109 may express its antiviral activity in vivo through the stimulation of the host's antiviral functions. When whole spleen cells (WSC), non-PAC (macrophage-depleted WSC), whole T cell preparations derived from non-PAC (WTC) or $CD8^+$ T cell preparations ($CD4^+$ T cell-depeleted WTC), which were prepared from spleens of mice treated with TJS-109, were adoptively transferred to mice (recipients) exposed to HSV-1, marked increases in resistance of recipient mice against the infection was demonstrated. However, the mortality rates stayed at 100% for recipient mice when they were inoculated with macrophage-enriched cells (PAC), B cell-enriched populations (nylon wool-adhered non-PAC fraction) or $CD4^+$ T cell populations ($CD8^+$ T cell-depleted WTC). These results suggest that the protective antiviral activities of TJS-109 may be expressed through the host functions mediating $CD8^+$ T cells.

The host's antiviral immune systems responding to HSV-1 include: generation of cytotoxic T cells (CTLs)[12,13], activation of natural killer cells[14], the production of humoral antibodies[15], the induction of antibody-dependent cellular cytotoxicity[16], and the production of various cytokines[14]. T lymphocytes have been reported to play a critical role in the protection and recovery of hosts infected with HSV-1[17,18]. The development of symptoms from HSV-1 infection was completely inhibited by the adoptive transfer of virus specific CTLs[19]. Our results presented here also indicate that $CD8^+$ T cells are involved in the anti-HSV-1 responses in mice treated with TJS-109. Further investigations into the details of the antiviral mechanism of TJS-109 are in progress.

There are many differences in treatments between Western drugs and Chinese herbal medicines. In Western medicine a diagnosis is made and a drug is chosen that has been proven effective for that disease[20]. Chinese herbal medicines on the other hand are chosen to treat the patients symptoms and the disease[20]. We are using current immunological methods to evaluate Chinese herbal medicines for their effectiveness in treating specific diseases. One example of this is demonstrated in

this paper describing the antiviral effect of TJS-109 that inhibited HSV-1 infection through the activation of the host's $CD8^+$ T cells. Therefore it may be possible for various other Chinese herbal medicines to be evaluated by recently developed immunological methods.

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