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Antiviral Research 32 (1996) 63–70

Antiviral Research

Prophylactic treatment of cytomegalovirus infection with traditional herbs

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Received 21 August 1995; accepted 2 June 1996

Abstract

Hot water extracts of four traditional herbs, *Geum japonicum*, *Syzygium aromaticum*, *Terminalia chebula* and *Rhus javanica*, which have been shown to have anti-herpes simplex virus (HSV) activity in vivo, were examined for anti-cytomegalovirus (CMV) activity in vitro and in vivo in this study. They inhibited replication of human CMV and murine CMV (MCMV) in vitro. These anti-CMV activities in vivo were examined in an MCMV infection model using immunosuppressed mice. Mice were subcutaneously treated with various doses of cyclosporine, and immunosuppression and MCMV infection were monitored by suppression of antibody production and virus yield in the lung, respectively. Each herbal extract was orally administered to mice treated with 50 mg/kg of cyclosporine from a day before intraperitoneal infection, and the efficacy of herbs was evaluated by the reduction in the virus yield in the lung. Among them *Geum japonicum*, *Syzygium aromaticum*, and *Terminalia chebula* significantly suppressed MCMV yields in lungs of treated mice compared with water treatment. Efficacy of oral treatment with 750 mg/kg per day of *Geum japonicum* extract was similar to that of the intraperitoneal administration of 2 mg/kg per day of ganciclovir in increasing the body weight of infected mice and reducing the virus yield in the lungs. These herbs may be beneficial for the prophylaxis of CMV diseases in immunocompromised patients.

Keywords: Cytomegalovirus; Antiviral activity; Herbs; Plant extract; Ganciclovir; Cyclosporine; Immunosuppression

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PII S0166-3542(95)00978-3

1. Introduction

Cytomegalovirus (CMV) infection is one of the troublesome infections in immunocompromised patients, especially transplant recipients and patients with acquired immunodeficiency syndrome (AIDS) (Ho, 1977; Marker et al., 1981; Betts and Hanshaw, 1977; Jacobson and Mills, 1988; Zaia, 1993). Symptomatic CMV infection has been successfully treated with ganciclovir, but the appearance of ganciclovir-resistant viruses is a current problem in the treatment of immunocompromised patients with CMV infection. Foscarnet has been used for combined treatment with ganciclovir and for the treatment of ganciclovir-resistant CMV, but is not always successful. New or alternative efficacious anti-CMV agents need to be developed (Barnard et al., 1993; Feng et al., 1992; Fong et al., 1987; Freitas et al., 1989; Ikeda et al., 1993; Neyts et al., 1993; Shigata et al., 1991; Snoeck et al., 1988; Stals et al., 1991, 1993; Yamamoto et al., 1990).

Traditional herbs have been used for the improvement of chronic diseases in the Asian countries and information on their efficacy and adverse reactions for long-term use have been accumulated for practical use (Jiangxu New Medical College, 1978). We screened about 150 herbal extracts for anti-herpes simplex virus (HSV), anti-measles, and anti-poliovirus activity in vitro and selected four herbs with therapeutic anti-HSV activity in vivo, *Geum japonicum*, *Syzygium aromaticum*, *Terminalia chebula* and *Rhus javanica* (Kurokawa et al., 1993, 1995). These four herbal extracts show therapeutic activity on cutaneous HSV infection and combined therapeutic activity with acyclovir. In addition they are also active against thymidine kinase-deficient HSV and phosphonoacetic acid-resistant HSV in vitro. Subsequently we extended the study of antiviral herbs to CMV infection. As interstitial pneumonia is one of the major targets of antiviral chemotherapy, we evaluated the anti-CMV activity in vivo of herbs by using lung infection (Osborn, 1982; Shanley and Pesanti, 1985) in mice immunosuppressed by cyclosporine (Land, 1987; Wilson et al., 1987). In this report we aimed to apply these herbs to the prophylactic treatment of CMV infection, and showed that one of the herbs, *Geum japonicum*, exhibited prophylactic anti-CMV activity as strong as ganciclovir.

2. Materials and methods

2.1. Cells and viruses

Mouse embryonic fibroblast (MEF) cells were prepared from ICR mice. ICR mice were purchased from Sankyo Laboratory Service. MEF and human embryonic lung (HEL) cells were grown and maintained in Eagle's minimum essential medium supplemented with 10 and 2% fetal bovine serum, respectively. MEF and HEL cells were plated in 60-mm plastic dishes for plaque titration. Murine CMV (Smith strain) was propagated in the MEF culture and used as an attenuated strain. MCMV and human CMV (HCMV, Towne strain) were harvested by three cycles of freezing and thawing of the infected cultures and centrifugation at 3000 rpm for 10 min, and stored at −85°C until use (Shiraki et al., 1990, 1991a,b).

2.2. Drugs

Cyclosporine (Sandimmun) and ganciclovir (Denosine) were purchased from Sandoz Pharmaceuticals and Tanabe Seiyaku, respectively.

Traditional herbs, *Geum japonicum* Thunb., *Syzygium aromaticum* (L) Merr. et Perry, *Terminalia chebula* Retzus and *Rhus javanica* L, were purchased from Tochimto-Tenkaido and authenticated. Their hot-water extracts were prepared as described previously (Kurokawa et al., 1993, 1995). Briefly, dried traditional herbs (100 g) were boiled under reflux in 1500 ml of distilled water for 3 h. These aqueous extracts were filtered and lyophilized. The lyophilized materials were suspended in distilled water at concentrations as described in the text. The suspension was boiled for 10 min and centrifuged for 15 min. Sterilized supernatant was used for the following assays.

2.3. Susceptibility of CMV to herbal extracts

The susceptibilities of HCMV to herbal extracts were evaluated by determining the effective concentration for 50% plaque reduction (EC_{50}: Kurokawa et al., 1993; Shiraki et al., 1990, 1991a,b). Briefly, confluent HEL cell monolayers (60-mm plastic dish) in duplicate or triplicate were infected with 100 plaque forming units (PFU) of HCMV for 1 h and incubated in the maintenance medium containing herbal extracts (0, 0.2, 0.5, 1, 2, 5, 10, 20, 50, and 100 mg/ml). After 10 days incubation, the cells were fixed with 5% neutral formalin solution and stained with methylene blue, and the number of plaques was counted with a dissecting microscope. The EC_{50} was determined graphically.

Anti-CMV activity of herbal extracts was also evaluated by the yield reduction of HCMV- or MCMV-infected cultures (Shiraki et al., 1991a,b; Kurokawa et al., 1995). HEL cell cultures in 25-cm^2 plastic flasks were infected with HCMV at 1 PFU/cell for 1 h and incubated for 3 days in the presence of 5 ml of maintenance medium containing the indicated concentrations (1, 3.2, 10, 32, 100 μg/ml) of herbal extracts. Then the cultures were frozen and thawed three times followed by centrifugation at 3000 rpm for 10 min and then their culture supernatants were subjected to the titration of the virus yields. Similarly, the MEF cultures were infected with MCMV in 25-cm^2 plastic flasks for 24 h and the infected cultures were incubated in the presence of herbal extracts. The virus yield was determined in the treated cultures. Anti-CMV activity of herbal extracts was expressed as the inhibitory concentration for 50% yield reduction (IC_{50}).

Cytotoxicity of herbal extracts was monitored by measuring their effect on the incorporation of [*methyl*-^{3}H]thymidine into the cellular DNA as reported previously (Kurokawa et al., 1993, 1995). Briefly, MEF and HEL cells were seeded at a concentration of 2.5×10^4 cells/well in 24-well plates and grown at 37°C for 2 days. The culture medium was replaced with fresh medium containing 74 kBq/ml of [*methyl*-^{3}H]thymidine (740 GBq/mmol, Amersham) and the indicated concentrations of the herbal extracts. After an 18-h exponential growth period of the cells, the cells were lysed with 20 mM Tris (pH 8.0), 5 mM EDTA, 0.5% SDS and 100 μg/ml of proteinase K at 37°C for 3 h. Acid-insoluble radioactivity was determined. Cytotoxicity of herbal extract was expressed as the cytotoxic concentration for 50% reduction in the incorporation into cellular DNA (CC_{50}).

2.4. Immunosuppression by treatment with cyclosporine

Antibody production to trinitrophenyl (TNP, Tokyo Kasei)–keyhole limpet hemocyanin (KLH, Calbiochem-Novabiochem) was compared in groups treated with various doses of cyclosporine (Good et al., 1980; Henry and Kimura, 1980). Ten female mice were injected subcutaneously with various doses of cyclosporine (3.2, 10, 32, 50, 100 mg/kg) once daily for 16 days starting 24 h before immunization, and they were intraperitoneally immunized with 20 μg of TNP–KLH mixed with 4 mg of alum. Antibody titer to TNP was assessed by the enzyme-linked immunosorbent assay on day 16 after immunization.

2.5. Cytomegalovirus infection in mice treated with cyclosporine

To evaluate anti-CMV activities of herbs in mice, we used MCMV grown in the MEF culture. Ten female ICR mice at the age of 4 weeks were injected subcutaneously with various doses of cyclosporine (3.2, 10, 32, 50, 100 mg/kg) once daily for 16 days starting 24 h before intraperitoneal infection with 1000 PFU of MCMV. The lungs were isolated under ether anesthesia and used for virus isolation and the determination of the virus titer on day 16 after infection. Ten percent homogenates of their lungs in phosphate buffered saline were serially diluted and infected in the MEF cultures overlaid with the nutrient methylcellulose medium. Virus titer in the lung was determined by counting the number of plaques in the cultures after 3 days incubation. The frequency of MCMV infection in the lung was deter-

mined by the number of mice with lung infection in the treated group. Toxicity of treatments was monitored by the increase in the body weight during this period.

2.6. Treatment of MCMV infection with herbs

The herbal extracts were orally administered with 5 mg/dose three times daily in 10 female ICR mice and this dose corresponded to that for human use (Kurokawa et al., 1993, 1995). Treatment with herbal extracts and immunosuppression by 50 mg/kg of cyclosporine were started daily from a day before infection. Mice were infected intraperitoneally with 1000 PFU of MCMV. Virus infection in the lung was evaluated on day 16 after infection as described above. Intraperitoneal administration with 1, 2.5, and 5 mg/kg of ganciclovir was performed twice a day from a day before infection to compare anti-CMV activity of ganciclovir with that of a herbal extract.

2.7. Histological examination of the lung

Lungs were isolated on day 16 after infection and fixed in 10% formalin solution. The specimens were dehydrated and embedded in paraffin. 4-μm-thick sections were cut and stained with hematoxylin and eosin and then examined by light microscopy (Nagasaka et al., 1995).

2.8. Statistical analysis

Data were analyzed using Student's t-test.

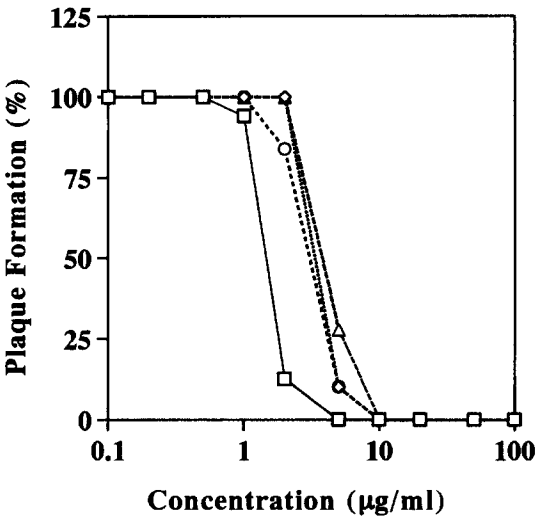

Fig. 1. Profile of anti-HCMV activity of herbal extracts in inhibition of plaque formation. Plaque formation was expressed by the percentage of untreated cultures. □, ○, ◇ and △ indicate *Rhus javanica*, *Terminalia chebula*, *Syzygium aromaticum* and *Geum japonicum*, respectively.

3. Results

3.1. Inhibition of HCMV growth in vitro by herbal extracts

Plaque formation of HCMV was dose-dependently inhibited by *Geum japonicum*, *Syzygium aromaticum*, *Terminalia chebula*, and *Rhus javanica* as shown in Fig. 1. The mean EC_{50} values in two experiments were 4.5, 4.3, 2.3 and 1.4 μg/ml for *Geum japonicum*, *Syzygium aromaticum*, *Terminalia chebula* and *Rhus javanica*, respectively. Table 1 shows the results of the yield reduction

Table 1
Susceptibility of HCMV and MCMV to herbal extracts and their cytotoxicity

Herbal extract	HCMV IC_{50}	HEL cells CC_{50}	MCMV IC_{50}	MEF cells CC_{50}
Geum japonicum	6.4	28	20.5	165
Syzygium aromaticum	7.6	42	18.0	71
Terminalia chebula	4.3	26	13.3	48
Rhus javanica	0.55	8.6	3.3	17.8

IC_{50}s (μg/ml) of herbal extracts for HCMV and MCMV were determined by the yield reduction assay in HEL and MEF cells, respectively. CC_{50} (μg/ml) of herbal extracts for HEL and MEF cells were determined by the cytotoxicity assay as described in the text.

assay of HCMV and MCMV by herbal extracts and the cytotoxicity assessed by the [^{3}H]thymidine uptake. Anti-HCMV or anti-MCMV activity was observed at the much lower concentration than the cytotoxic concentration in each herbal extract. Herbal extract showed anti-CMV activity in both plaque reduction and yield reduction assays.

3.2. MCMV infection in the lungs of mice treated with cyclosporine

In the time course study, the frequency of infection and virus yield in the lung increased with time until 16 days after infection and decreased thereafter (data not shown). We therefore evaluated MCMV infection in the lung on day 16 after infection. The results on the frequency of infection and virus yield in the lung are summarized as follows. The frequency of infection in the lung increased dose-dependently with cyclosporine. The dose attaining the frequency of 50% infection was 5.4 mg/kg for cyclosporine. Virus yields in the lungs increased dose-dependently with cyclosporine. All mice were infected and their virus yields in the lung were high enough to evaluate the effect of drugs on lung infection in mice treated with 50 mg/kg cyclosporine. CMV infection was not lethal in this condition.

3.3. Determination of dose of cyclosporine for immunosuppression

Cyclosporine suppressed the production of antibody to TNP dose-dependently (data not shown). The effective dose for 50% reduction (ED_{50}) in antibody production was 11 mg/kg for cyclosporine. Treatment with 50 mg/kg and more of cyclosporine inhibited antibody production almost completely.

The level of immunosuppression was monitored by antibody production to TNP–KLH as described above, and the lung infection was evaluated by the frequency of infection and the virus titer in the lung. As stated above, treatment with 50 mg/kg of cyclosporine inhibited antibody production, and the lungs of all mice were infected with CMV in this condition. Increase in body weight of mice treated with and without cyclosporine was 3.56 ± 0.94 and 5.40 ± 0.91 g, respectively. Thus treatment of mice with 50 mg/kg of cyclosporine significantly suppressed the gain of body weight in infected mice ($P < 0.01$) but was not lethal to infected mice. Therefore the dosage of 50 mg/kg per day of cyclosporine was used to evaluate the efficacy of traditional herbs in prophylactic treatment of MCMV infection.

Table 2
Effect of herbal extracts on the virus yields in the lungs of immunosuppressed mice

Treatments	Virus yield in the lung
Water	5.68 ± 2.85
Geum japonicum	1.88 ± 1.13 ($P<0.001$)[a]
Syzygium aromaticum	3.35 ± 3.15 ($P<0.05$)[a]
Terminalia chebula	2.24 ± 2.22 ($P<0.001$)[a]
Rhus javanica	6.70 ± 8.18

Oral treatment with herbal extracts (250mg/kg × 3/day) was started 1 day before intraperitoneal MCMV infection (1000 PFU) in mice immunosuppressed with 50 mg/kg of cyclosporine. Virus titers were expressed as the mean ± S.D. ($\times 10^3$) PFU/0.1 g of the lung tissue ($n = 10$).
[a] Comparison with water treatment.

3.4. Prophylactic treatment of MCMV infection in the lung with herbal extracts

Table 2 shows the virus yields in the lungs of immunosuppressed mice treated with herbal extracts. *Geum japonicum*, *Syzygium aromaticum*, and *Terminalia chebula* significantly suppressed CMV yields in lungs of cyclosporine-treated mice compared with water treatment. *Rhus javanica* was not effective in reducing the virus yield in the lung of immunosuppressed mice. These results were confirmed by repeating experiments. *Geum japonicum* strongly suppressed the virus growth in the lung and its anti-CMV activity was further examined. Treatment with *Geum japonicum* daily from 1 day before and just after infection was effective in reducing virus yield in the lung, but that with *Geum japonicum* daily from 1 day after infection was not effective as shown in Table 3. Table 4 shows the comparison of anti-CMV activity of oral *Geum japonicum* treatment with ganciclovir treatment. The efficacy of *Geum japonicum* in reducing virus yields in the lung was similar to

Table 3
Effect of interval between oral *Geum japonicum* treatment and MCMV infection

Treatment	Virus yield in the lung
Water	1.97 ± 0.48
1 day before infection	1.00 ± 0.95 ($P<0.01$)[a]
Same day	1.14 ± 0.52 ($P<0.01$)[a]
1 day after infection	2.00 ± 2.12

Oral *Geum japonicum* treatment was started 1 day before, the same day as, and 1 day after MCMV infection. Doses of *Geum japonicum* and cyclosporine were the same as those of Table 1. Virus titers were expressed as the mean ± S.D. ($\times 10^3$) PFU/0.1 g of the lung tissue ($n = 10$).
[a] Comparison with water treatment.

that of intraperitoneal treatment with 2 mg/kg per day of ganciclovir and there was no statistically significant difference between them. Toxicity of herbal extracts was assessed by the increase in body weight. Treatments of infected mice with *Geum japonicum* and ganciclovir significantly increased their body weight compared with water treatment ($P < 0.05$ and 0.01, respectively) and no significant difference in the increase in body weight was observed among treatment groups with *Geum japonicum* and ganciclovir ($P > 0.05$). Thus oral *Geum japonicum* treatment with 750 mg/kg per day in three doses significantly reduced the virus yields in the lungs and its anti-MCMV activity corresponded to the intraperitoneal treatment with 2 mg/kg per day of ganciclovir in two doses.

3.5. Histopathological study

Inflammatory responses were not observed in the lung of infected mice and there was no significant difference in the appearance of histopathologic lesions in the lungs of water- and herb-treated mice. This may be caused by the strong immunosuppression with cyclosporine.

4. Discussion

HCMV infection has been treated with ganciclovir, foscarnet, immunoglobulin, etc. but the treatment is not always successful in immunocompromised hosts. Herbal extracts were first examined for anti-HCMV activity in HEL cells and all inhibited the plaque formation at 1–5 μg/ml of the herbal extract. These anti-CMV activities were further assessed by the yield reduction assays of HCMV and MCMV. We used the immunosuppressed mouse system for CMV infection to analyze the anti-CMV activity in vivo of herbal extracts (Ikeda et al., 1993; Neyts et al., 1993; Stals et al., 1993; Wilson et al., 1987). The lung is one of the major targets for CMV infection in humans and mice, and we selected lung infection as the marker of the severity of infection (Osborn, 1982; Shanley and Pesanti, 1985; Zaia, 1993). Virus titer in the liver was lower than that in the lung. We used the attenuated MCMV grown in the tissue culture (Osborn, 1982) and the mouse

Table 4
Comparison of oral *Geum japonicum* treatment with intraperitoneal ganciclovir treatments

Treatments	Dose (mg/kg)	Virus yield in the lung	Increase in body weight
Water		5.41 ± 3.40	2.86 ± 1.05
Geum japonicum	250 (×3/day)	0.90 ± 1.11[a,b,c]	4.16 ± 1.23[d]
Ganciclovir	5 (×2/day)	0.019 ± 0.24[a]	4.66 ± 1.39[a]
	2.5 (×2/day)	0.078 ± 0.014[a]	4.21 ± 1.36[d]
	1 (×2/day)	0.79 ± 0.41[a]	5.64 ± 1.78[a]

Virus titers were expressed as the mean ± S.D. ($\times 10^3$) PFU/0.1 g of the lung tissue ($n = 10$). Increase in body weight during treatment was expressed as the mean ± S.D. g in the same treatment group.
[a] $P<0.001$ vs. water treatment.
[b] $P = 0.499$ vs. ganciclovir treatments with 1 mg/kg × 2/day.
[c] $P<0.01$ vs. ganciclovir treatments with 5 and 2.5 mg/kg × 2/day.
[d] $P<0.05$ vs. water treatment.

system under immunosuppression with 50 mg/kg per day of cyclosporine for the antiviral assay system. Thus, the virus yields in the lung were used to evaluate the anti-CMV efficacy of herbal extracts, because all mice were infected and the virus titer in the lung was quantifiable. Oral administration of these herbal extracts for 7 days were not toxic to mice even in combination with 5 mg/kg three times daily as reported previously (Kurokawa et al., 1995).

Traditional herbs have been used for the improvement of chronic diseases in the Asian countries and the information on their efficacy and adverse reactions for long-term use have been accumulated for practical use (Jiangxu New Medical College, 1978; Kurokawa et al., 1993). Therefore, we assumed that these traditional herbs might be applicable as prophylactic drugs to prevent CMV diseases in immunocompromised hosts, because these herbs have been used for the treatment of chronic diseases without major adverse reactions in the prolonged administration as described above. On these bases, we elucidated the efficacy of the traditional herbs in the prophylactic treatment against CMV infection.

Although the therapeutic basis or clinical efficacy has not been so well established as those of chemically defined drugs, we have shown the therapeutic efficacy of the traditional herbs against HSV infection in mice (Kurokawa et al., 1993, 1995; Nagasaka et al., 1995). Four traditional herbs have been shown to be effective in the treatment of experimental HSV-1 infection in mice and exhibited combined therapeutic efficacy with acyclovir. Their anti-HSV-1 action was not affected by acyclovir resistance. *Geum japonicum*, *Syzygium aromaticum*, and *Terminalia chebula* were effective in reducing the virus yields in the lung but *Rhus javanica* was not effective, suggesting the specificity of its antiviral action to HSV.

In this study, oral treatment with *Geum japonicum* at the dose corresponding to human use exhibited anti-CMV activity as effective as the intraperitoneal treatment with 2 mg/kg per day of ganciclovir. Wilson et al. (1987) reported that ganciclovir treatment with 2.5 and 1.25 mg/kg per day resulted in 40 and 25% survival, respectively, in the lethal MCMV infection. Freitas et al. (1989) reported that 26.6 and 28.5% of mice survived by treatment with 1 and 3 mg/kg per day of ganciclovir in the lethal MCMV infection in mice. Therefore, the anti-CMV activity of oral *Geum japonicum* treatment may be promising, based on the anti-CMV activity of the corresponding dose of ganciclovir. In conclusion, three traditional herbs which have been historically used for the improvement of chronic diseases, were proven to be effective for the prophylactic treatment of CMV infection.

Acknowledgements

This work was supported in part by a Grant-in-Aid from the Ministry of Human Health and Welfare, the Research Committee on Prevention of Developing Illnesses and Therapy for HIV-infected Patients. We thank Ms Tomoko Kamiyama and Tomoko Okuda for their excellent technical assistance.

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