Efficacy of Kampo medicines on cutaneous herpes simplex virus type 1 infection in mice

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Abstract

A cutaneous herpes simplex virus type 1 (HSV-1) infection model in mice has been shown to be useful in defining the biological activity of traditional medicines because there are various markers in the model to evaluate their therapeutic efficacy. Using this murine model as a model for human virus infection, we examined and characterized the therapeutic anti-HSV-1 efficacy of 32 Kampo medicines which have been commonly used for the treatment of various diseases in China and Japan. None of these showed anti-HSV-1 activity in the plaque reduction assay. Mice were cutaneously infected with HSV-1 and orally administered with the extracts of Kampo medicines three times daily for 7 days at doses corresponding to human use. Kumi-binlo-to (九味檳榔湯), Shishi-hakuhi-to (梔子柏皮湯) and Anchu-san (安中散) significantly retarded the development of skin lesion, and Syo-saiko-to (小柴胡湯), Saiko-keishi-to (柴胡桂枝湯) and Gingyo-san (銀翹散) were significantly effective in prolonging the mean survival times of the infected mice as compared with water-administered mice. When cutaneous reaction representing delayed-type hypersensitivity (DTH) to HSV-1 antigen was examined in the infected mice treated with these effective Kampo medicines, 4 of them prominently augmented DTH reaction as compared with the mice treated with water. Since DTH is induced by macrophage and Th1 cells and it is also the major host defense mechanism for cutaneous HSV infection, DTH reaction augmented by these Kampo medicines may mainly contribute to the therapeutic efficacy without direct anti-HSV activity *in vitro*. This novel biological activity of Kampo medicines is discussed and these results may contribute to analyzing their pharmacological actions.

Key words Kampo medicines, herpes simplex virus, antiviral activity, delayed-type hypersensitivity, infectious disease, skin lesion.

Abbreviations DTH, delayed-type hypersensitivity ; EC_{50}, effective concentration for 50 % plaque reduction ; HSV-1, herpes simplex virus type 1 ; HW-extracts, hot-water extracts ; MEM, Eagle's minimum essential medium ; PFU, plaque forming unit ; Th1, T helper 1 cell.

Introduction

We have been studying the therapeutic efficacies of medicinal herbs using a cutaneous herpes simplex virus type 1 (HSV-1) infection model in mice because of the presence of various markers in the development of diseases,[1,2] and this model has been shown to have advantages for monitoring efficacies of Kampo medicine. Using this murine cutaneous infection model, we have previously selected 12 from 142 medicinal herbs, which exhibit anti-HSV-1 activity *in vitro* and *in vivo*.[2] Kakkon-to, a Kampo formulation, which is composed of 7 herbs exhibits therapeutic anti-HSV-1 efficacy in the murine model by enhancing delayed-type hypersensitivity (DTH) response against HSV-1 antigen as assessed by cutaneous reaction.[1] Kampo medicines are popular medicines and can be easily

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obtained in China and Japan. They have been safely and cautiously managed for the treatment of various diseases based on the information of adverse reactions accumulated historically in traditional therapy.[3,4] Thus we chose 32 Kampo medicines to study their biological activity and therapeutic efficacy in the murine infection model. These Kampo medicines include at least one of these 12 effective herbs [2] and have been used for the improvement of various symptoms that are probably caused by viral infection. In this study four of the 32 Kampo medicines exhibited therapeutic efficacy against HSV-1 infection by augmenting significantly the DTH reaction against HSV-1 antigen. Thus we have shown one of the pharmacological activities of Kampo medicines in this established murine HSV infection model and this may contribute to the further analysis of their pharmacological actions.

Materials and Methods

Virus and cells : HSV-1 7401H strain was propagated in Vero cells.[5,6] The infected cultures were frozen and thawed three times and centrifuged at 3,000 rpm for 15 min. Their supernatants were stored at −80°C until use.[5,6] Vero cells were grown and maintained in Eagle's minimum essential medium (MEM) supplemented with 5 % and 2 % calf serum, respectively.

Preparation of herbal extracts (*Tables I and II*) : Tokaku (桃核 ; Tao-He, 1 in Tables), Saiboku-to (柴朴湯 ; Chai-Pu-Tang, 2), Ryokei-jutsu-kan-to (苓桂朮甘湯 ; Ling-Gui-Shu-Gan-Tang, 3), Juzen-taiho-to (十全大補湯 ; Shi-Quan-Da-Bu-Tang, 4), Kumi-binlo-to (九味檳榔湯 ; Jiu-Wei-Bing-Lang-Tang, 5), San'o-shashin-to (三黄瀉心湯 ; San-Huang-Xie-Xin-Tang, 6), Shishi-hakuhi-to (梔子柏皮湯 ; Zhi-Zi-Bai-Pi-Tang, 7), Saiko-seikan-to (柴胡清肝湯 ; Chai-Hu-Qing-Gan-Tang, 8), Anchu-san (安中散 ; An-Zhong-San, 9), Sansounin-to (酸棗仁湯 ; Suan-Zao-Ren-Tang, 10), Kikyo-to (桔梗湯 ; Jie-Geng-Tang, 11), Jumi-haidoku-to (十味敗毒湯 ; Shi-Wei-Bai-Du-Tang, 12), Ji-daboku-ippo (治打撲一方 ; Zhi-Da-Pu-Yi-Fang, 13), Tokaku-joki-to (桃核承気湯 ; Tao-He-Cheng-Qi-Tang, 14), Oren-gedoku-to (黄連解毒湯 ; Huang-Lian-Jie-Du-Tang, 15), Hange-shashin-to (半夏瀉心湯 ; Ban-Xia-Xie-Xin-Tang, 16), Nyoshin-san (女神散 ; Nu-Shen-San, 17), Syo-saiko-to (小柴胡湯 ; Xiao-Chai-Hu-Tang, 18), Kami-shoyo-san (加味逍遙散 ; Jia-Wei-Xiao-Yao-San, 19), Dai-saiko-to (大柴胡湯 ; Da-Chai-Hu-Tang, 20), Otsuji-to (乙字湯 ; Yi-Zi-Tang, 21), Keigai-rengyo-to (荊芥連翹湯 ; Jing-Jie-Lian-Qiao-Tang, 22), Hochu-ekki-to (補中益気湯 ; Bu-Zhong-Yi-Qi-Tang, 23) were all supplied by Showa Shell Sekiyu, K. K. ; Syo-saiko-to (26 and 27, two different lots), Saiko-keishi-to (柴胡桂枝湯 ; Chai-Hu-Gui-Zhi-Tang, 28 and 29, two different lots), Gingyo-san (銀翹散 ; Yin-Qiao-San, 30 and 31, 2 different lots ; 32, tablet made in China), and Kakkon-to (葛根湯, 24 and 25, two different lots which served as active controls in Table II) were prepared as hot water (HW)-extracts.[1,2] All these extracts of Kampo medicines were suspended in water, boiled for 10 min, and centrifuged for 15 min as described previously.[1,2] The supernatants were used for *in vitro* and *in vivo* assays.

Plaque reduction assay : HW-extracts of Kampo medicines were examined for their anti-HSV-1 activity by the plaque reduction assay. Duplicate cultures of Vero cells in 60 mm plastic dishes were infected with 100 plaque forming units (PFU)/0.2 ml of HSV-1 for one hour. Then the cells were overlaid with 5 ml nutrient methylcellulose (0.8 %) medium containing HW-extracts at 100, 300, 500, and 700 μg/ml. A medium control was included in each assay. After 3-day incubation of infected cultures at 37°C, the cells were fixed with formalin and stained with methylene blue, and the number of plaques was counted.[5,6] The 50 % effective concentrations (EC_{50}) for plaque reduction of HW-extracts were determined from curves relating the plaque reduction percentages to the concentrations of HW-extracts.[2]

Murine HSV-1 infection model : Six or seven-week-old female BALB/c mice were purchased from Sankyo Lab. Service Co., Ltd., Tokyo, Japan. The right midflank of each mouse was clipped and depilated with a chemical depilatory (hair remover, Shiseido Co., Ltd., Tokyo, Japan). The hairless skin was scratched with a bundle of 27-gauge needles,[1,2,7,8] giving a total scarified area of approximately 1.0 cm^2 and was immediately infected by spreading a 10 μl drop of virus suspension containing 1 $\times 10^6$ PFU, resulting in

the superficial infection of the dermal tissue. HW-extracts were orally administered to mice at doses as indicated once 4 hours before and three times daily for 7 successive days after infection (Table II). The dosages used for mice corresponded to the conventional dose of each Kampo medicine for humans. Water-treated mice with the same infection were used as a control. Skin lesion development and mortality rate were continuously monitored 3 times daily and scored as described previously.[1,2] Briefly, 0, no lesion ; 2, vesicles in local region ; 4, erosion and/or ulceration in local region ; 6, mild zosteriform lesion ; 8, moderate and severe zosteriform lesion ; 10, death. The infected mice were observed for about one month to determine their mortality. The therapeutic efficacy was expressed by the mean time of appearance of scores 2 and 6, mean survival times and mortality.

Induction of cutaneous reaction to HSV-1 antigen in mice : Five of the 6 Kampo medicines showing therapeutic anti-HSV-1 efficacy were examined for their action on cutaneous reaction against HSV-1 antigen in infected mice (Anchu-san was not examined since the amount of HW-extract was not enough for oral administration in the skin test experiment). Mice were injected intradermally (within dermal tissue) with HSV-1 antigen and administered orally with HW-extracts as described above. Under ether anesthesia, the infected mice were challenged by intradermal injection of 10 μl containing 1×10^6 PFU of ultraviolet light-inactivated virus into the right footpad of each mouse on day 4 after infection as described elsewhere.[1,9] Left footpads were not challenged. Then the induced local swelling of footpads was measured at 24, 36 and 48 hours after the challenge by using an engineer's micrometer. Water-administered mice were used as control.

Statistical analysis : Student's t-test was used to evaluate the statistical differences in mean survival times and mean times at which skin lesions were initially scored as 2 and 6 after infection as well as cutaneous reaction. The differences between cutaneous reaction in water- and HW-extract-treated mice were also examined by the Student's t-test. Statistical differences in the mortality rate were evaluated using Fisher's exact test. A P-value of less than 0.05 was statistically defined as significant.

Results

Anti-HSV-1 activity in vitro

The inhibitory activity of 32 Kampo medicines on the plaque formation of HSV-1 infected Vero cells was examined in order to evaluate their direct antiviral activity (Table I). The EC_{50} values of all HW-extracts examined were more than 500 or 700 μg/ml. No HW-extracts used exhibited potent anti-HSV-1 activity. Visible cytotoxicity was not found in all 32 HW-extract treated cells.

Effects of Kampo medicines on HSV-1 infected mice

HW-extracts of 32 Kampo medicines including their different lots were examined for their potential therapeutic efficacy against HSV-1 infection in a cutaneously HSV-1 infection model in mice. The mean times at which vesicles and erosion were initially appeared, mean survival times and mortality rate are summarized in Table II. The 2 different lots (24 and 25) of Kakkon-to used as active controls significantly retarded skin lesions and/or prolonged mean survival times as compared with water-administered mice. Kumi-binlo-to (5), Shishi-hakuhi-to (7), and Anchu-san (9) were significantly effective in

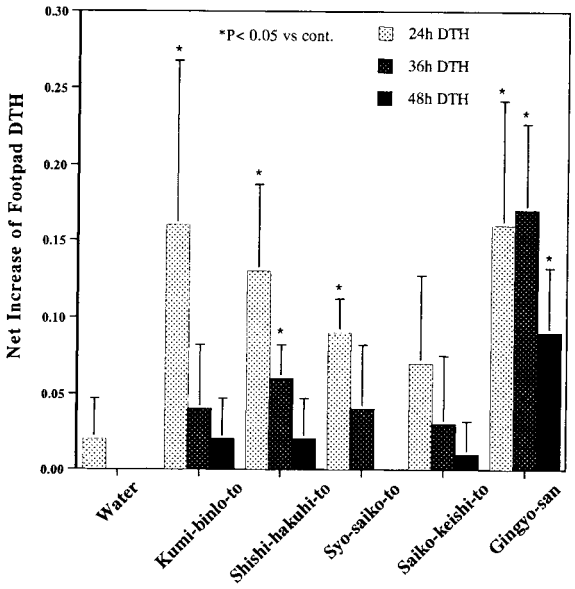

Fig. 1 Time course of cutaneous reaction to HSV-1 antigen in mice infected intradermally with HSV-1. Each column and bar represents the mean swelling of 10 mouse-footpads and S.D. respectively. *$p<0.05$ vs control by Student's t-test.

Table I *In vitro* anti-HSV-1 assay of Kampo medicines

No.	Kampo medicines	% plaque formation				EC_{50}
		Concentrations (μg/ml)				
		100	300	500	700	
1	Tokaku	94.2	79.2	59.2	ND	>500
2	Saiboku-to	100.2	71.5	66.2	ND	>500
3	Ryokei-jutsu-kan-to	88.2	89.2	85.3	ND	>500
4	Juzen-taiho-to	100.0	90.1	94.6	ND	>500
5	Kumi-binlo-to	101.5	96.2	90.0	ND	>500
6	San'o-shashin-to	93.1	84.6	86.2	ND	>500
7	Shishi-hakuhi-to	94.6	90.4	86.2	ND	>500
8	Saiko-seikan-to	93.1	94.6	101.5	ND	>500
9	Anchu-san	ND	ND	ND	ND	
10	Sansonin-to	91.2	103.1	99.2	ND	>500
11	Kikyo-to	81.5	84.2	93.8	ND	>500
12	Jumi-haidoku-to	92.4	110.5	97.4	ND	>500
13	Ji-daboku-ippo	110.5	72.0	66.7	ND	>500
14	Tokaku-joki-to	109.5	106.2	96.2	ND	>500
15	Oren-gedoku-to	112.4	108.6	105.7	ND	>500
16	Hange-shashin-to	106.7	103.8	107.6	ND	>500
17	Nyoshin-san	100.0	100.0	100.1	ND	>500
18	Syo-saiko-to	118.1	100.0	99.4	ND	>500
19	Kami-shoyo-san	110.5	122.9	90.5	ND	>500
20	Dai-saiko-to	101.9	103.5	101.9	ND	>500
21	Otsuji-to	102.9	107.6	99.0	ND	>500
22	Keigai-rengyo-to	104.8	105.7	89.5	ND	>500
23	Hochu-ekki-to	115.2	83.8	78.1	ND	>500
24	Kakkon-to, 551010	98.1	93.8	87.0	ND	>500
25	Kakkon-to, 11G	95.6	93.1	91.3	ND	>500
26	Syo-saiko-to, 571810	91.3	93.8	91.9	ND	>500
27	Syo-saiko-to, 551611	90.1	88.8	91.8	ND	>500
28	Saiko-keishi-to, 19F	92.5	95.0	92.6	ND	>500
29	Saiko-keishi-to, 29F	95.0	92.5	89.8	ND	>500
30	Gingyo-san, 3026	ND	ND	91.6	84.7	>700
31	Gingyo-san, 3027	ND	ND	91.6	94.9	>700
32	Gingyo-san tablet (China)	ND	ND	105.1	108.3	>700

ND, not detected

retarding the development of skin lesions, and Syo-saiko-to (26, 27), Saiko-keishi-to (28, 29) and Gingyo-san (30, 31 and 32) were significantly effective in prolonging the mean survival times. Thus these 6 kinds of Kampo medicines were therapeutically effective against HSV-1 infection in the murine infection model as assessed by the retardation of skin lesion development and progression, or prolongation of survival time.

Effect of Kampo medicines on cutaneous reaction to HSV-1 antigen

Five of the 6 effective HW-extracts of Kampo medicines were examined for their cutaneous reactions to HSV-1 antigen in HSV-1 infected mice. Intradermal application of HSV-1 antigen induced the cutaneous reaction in infected mice (Fig. 1). Cutaneous reaction in right footpads was stronger in mice treated with HW-extract than those treated with water. No visible changes were observed in left footpads. Kumi-binlo-to (5), Shishi-hakuhi-to (7),

Table II Effects of HW-extracts of Kampo medicines on cutaneous HSV-1 infection in BALB/c mice.

Expt. No.	Treatment (mg/kg/day)[a]	Mean time (days±S.D.) Score 2[b]	Score 6[b]	Survival[c]	Mortality[d]
Expt. 1					
	Control (water)	4.00±0.50	6.13±0.35	7.88±1.46	9/9
	1. Tokaku (123.2)	4.30±1.06	5.67±0.52	7.17±0.75	7/10
	2. Saiboku-to (740.0)	4.40±0.70	5.67±0.82	6.67±0.82	7/10
	3. Ryokei-jutsu-kan-to (360.0)	3.78±0.67	6.33±0.82	6.60±0.89	7/8
	4. Juzen-taiho-to (1340.0)	4.30±0.68	5.67±0.52	7.67±0.82	7/10
	5. Kumi-binlo-to (675.5)	5.00±0.67[e]	7.50±1.64[e]	7.50±0.58	6/10
	6. San'o-shashin-to (245.0)	4.00±0.71	6.00±0.00	7.44±1.33	9/9
	7. Shishi-hakuhi-to (260.0)	4.20±0.84	6.75±0.50[e]	7.75±0.50	4/5
	8. Saiko-seikan-to (1060.0)	4.44±0.73	6.40±0.89	7.50±1.23	7/9
	9. Anchu-san (410.0)	5.00±1.05[e]	6.00±1.16	6.17±0.98	7/10
	10. Sansonin-to (550.0)	4.22±0.67	6.00±0.63	7.88±1.73	8/10
	11. Kikyo-to (415.0)	4.00±0.82	6.00±0.00	7.75±0.50	4/4
Expt. 2					
	Control (water)	3.40±0.52	5.78±0.44	6.90±0.74	10/10
	12. Jumi-haidoku-to (910.0)	3.78±0.44	6.00±0.00	6.67±0.50	9/9
	13. Ji-daboku-ippo (400.0)	3.60±0.52	5.90±0.32	6.50±0.71	10/10
	14. Tokaku-joki-to (355.0)	3.40±0.52	5.43±0.54	6.40±0.52	10/10
Expt. 3					
	Control (water)	3.50±0.53	5.00±0.00	7.10±0.74	10/10
	15. Oren-gedoku-to (400.0)	3.67±0.50	5.00±0.00	7.00±1.00	9/9
	16. Hange-shashin-to (890.0)	3.67±0.58	5.00±0.00	6.33±0.58	3/3
	17. Nyoshin-san (1155.0)	3.40±0.52	5.00±0.00	7.20±0.79	10/10
	18. Syo-saiko-to (1340.0)	3.67±0.50	5.00±0.00	6.78±0.67	9/9
	19. Kami-shoyo-san (1125.0)	3.89±0.33	5.00±0.00	6.50±0.54	8/8
	20. Dai-saiko-to (1355.0)	3.44±0.53	5.11±0.33	7.25±0.89	8/8
	21. Otsuji-to (665.0)	3.78±0.44	5.00±0.00	6.67±0.71	9/9
	22. Keigai-rengyou-to (1115.0)	3.44±0.53	5.00±0.00	7.33±1.12	9/9
	23. Hochu-ekki-to (1520.0)	3.30±0.68	5.00±0.00	7.30±1.06	10/10
Expt. 4					
	Control (water)	3.20±0.42	5.10±0.32	6.20±0.42	10/10
	24. Kakkon-to, 551010 (1040.0)	3.50±0.53	5.40±0.70	6.90±0.74[e]	10/10
	25. Kakkon-to, 11G (1040.0)	3.67±0.50[e]	5.22±0.67	7.33±0.87[e]	10/10
	26. Syo-saiko-to, 571810 (1080.0)	3.33±0.50	5.22±0.67	7.56±0.53[e]	10/10
	27. Syo-saiko-to, 551611 (1080.0)	3.30±0.48	5.20±0.42	6.90±0.32[e]	10/10
	28. Saiko-keishi-to, 19F (800.0)	4.25±0.71[e]	5.75±1.04	7.29±1.38[e]	9/10
	29. Saiko-keishi-to, 29F (800.0)	3.56±0.88	5.56±1.01	7.00±1.00[e]	10/10
	30. Gingyo-san, 3026 (2140.0)	3.43±0.54	5.14±0.90	7.14±0.69[e]	10/10
	31. Gingyo-san, 3027 (2140.0)	3.63±0.74	5.88±1.13	7.14±0.69[e]	9/10
	32. Gingyo-san, China (1290.0)	3.60±0.52	5.00±0.47	7.11±0.78[e]	9/10

[a]Dosage for administration for three times daily
[b]Mean time at which score 2 or 6 was first observed after infection
[c]Surviving mice were not included for the calculation of mean survival times.
[d]Number of dead mice/number of mice tested
[e]$p<0.05$ vs. control by Student's t-test

Syo-saiko-to (26), and Gingyo-san (31) significantly augmented cutaneous reaction at 24, 36 and/or 48 hours after challenge with HSV-1 antigen, but Saiko-keishi-to (28) did not. Thus four of six Kampo medicines which exhibited therapeutic efficacy augmented cutaneous reaction to HSV-1 antigen.

Discussion

We examined the therapeutic anti-HSV-1 activity of 32 Kampo medicines in a murine cutaneous HSV-1 infection model. This model has been shown to be helpful in evaluating the therapeutic efficacy of the medicines against human HSV-1 infection and would be able to be utilized as a model for defining biological activity of Kampo medicines.[1,2] Among the 32, Kumi-binlo-to, Anchu-san, Saiko-keishi-to, Shishi-hakuhi-to, Syo-saiko-to, and Gingyo-san were found to be active medicines against cutaneous HSV-1 infection, which is equivalent or more than 5 mg/kg acyclovir treatment in this murine model.[2] Most of these six Kampo medicines significantly augmented the cutaneous reaction to HSV-1 antigen. Strong cutaneous reaction continued longer than 24 hours after challenge with HSV-1 antigen and this indicated that cutaneous reaction represented the DTH reaction to HSV-1 antigen. It has been reported that DTH reaction is a major defense in intradermal HSV-1 infection.[1,9,10] Kampo medicines used in this study did not cause the reduction of plaque formation, but there is a possibility that the metabolites of herbal extracts might have an anti-HSV-1 activity, especially Saiko-keishi-to which did not augment DTH significantly. Yet this is the first report about the therapeutic efficacy and biological activity of these six Kampo medicines to HSV-1 cutaneous infection; and this biological activity may contribute to the clarification of their pharmacological basis.

Nonspecific defense against HSV-1 infection, such as natural killer cell activity, natural cytotoxic cell activity and the population of T-cell subsets in spleen and interferon activity in serum, is not affected by Kakkon-to compared with water-treated mice. Specific humoral immunity to HSV-1 is not affected in Kakkon-to-treated mice. Kakkon-to induces strong DTH reaction against HSV-1 antigen and its enhancement results in the therapeutic efficacy in the cutaneously HSV-1 infected mice.[1] Based on these we examined the cutaneous reaction to HSV-1 antigen in Kampo medicines-administered mice in this study. Kumi-binlo-to, Shishi-hakuhi-to, Syo-saiko-to, and Gingyo-san prominently augmented the DTH as assessed by cutaneous reaction against HSV-1 antigen and prolonged the mean surival time efficiently. The augmentation of DTH reaction to HSV-1 antigen may also be similar to that by Kakkon-to, resulting in therapeutic efficacy in retarding the skin lesion development. Macrophages and Th1 cells play essential roles in the induction of DTH response[11,12] and this indicates that Kampo medicines may have activated these biologically important cells against HSV-1 antigens and induced DTH strong enough to exhibit therapeutic efficacy. Thus this study clarified one of the targets or actions of the Kampo medicines, the activation of macrophages and/or Th1 cells.

Kampo medicines have been used historically for the treatment of various diseases or symptoms. Their dosages and application have been selected and established by balancing possible efficacy and the adverse reactions in their history. In this study, we used the dosage of each Kampo medicine for mice corresponding to the conventional dosage for humans. These six kinds of Kampo medicines have been used clinically for the treatment of myocardial dysfunction, nephrosis, abdominal pain, pain of the stomach regurgitation, acute fever, sweating, icterus, pruritus, nausea, common cold and pneumonia, or auxiliary treatment of tumors, *etc.*[3,4,13,14] We have shown the novel biological action, the augmentation of DTH to HSV-1 antigen, of Kampo medicines in murine HSV infection model and activation of macrophages and/or Th1 cells may contribute to understanding and analyzing their pharmacological actions.

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和文抄録

マウスを用いた単純ヘルペスウイルス1型 (HSV-1)

皮膚感染実験系には治療効果を検討できる種々のマーカーがあり，伝統医薬の生物活性を明らかにするために有効な *in vivo* 実験系である。そこで，この感染実験系を用い，32種の漢方薬のHSV-1感染症に対する治療効果とその生物活性を検討した。用いた32種の方剤は，*in vitro* でのHSV-1プラック形成阻止活性を認めなかった。続いて，HSV-1経皮感染マウスにヒトの投与量に相当する漢方薬熱水エキスを1日3回，7日間経口投与し，マウスHSV-1感染実験系における方剤の治療効果を検討した。九味檳榔湯，安中散，梔子柏皮湯及び柴胡桂枝湯はHSV-1感染マウスの皮膚病変の出現と進展を有意に遅延し，小柴胡湯，柴胡桂枝湯及び銀翹散は生存時間を有意に延長した。さらに，九味檳榔湯，梔子柏皮湯，小柴胡湯，銀翹散は，水投与マウスに比べてHSV-1抗原に対して遅延型過敏反応（DTH）を有意に増強した。これらのことから，これら漢方薬はDTHの誘導に関与するマクロファージ・Th1リンパ球を活性化することにより，DTHを含む生体特異的免疫機能を増強し，皮膚病変を遅延させ，生存時間をも延長したと考えられた。以上のように，6種の方剤の生物活性がHSV-1皮膚感染実験系の治療効果とDTHの増強効果により確認できた。このことはこれら方剤の薬効と作用の解析に重要な示唆を与えた。

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