Antipyretic Activity of Gingyo-san, a Traditional Medicine, in Influenza Virus-Infected Mice

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Gingyo-san is composed of 10 crude drugs and used as a traditional antipyretic medicine for the treatment of the common cold and influenza virus infection. In a murine intranasal influenza infection model, fever produced by the infection has been demonstrated to be reduced by suppressing interferon-induced interleukin (IL)-1α production. Thus, we focused on the serum level of IL-1α which produces such novel antipyretic activity, and evaluated the relationship between defervescence and the suppression of IL-1α production by Gingyo-san in influenza virus-infected mice. Fever was produced in the infected mice 33—44 h after infection. Oral administration of a hot water-extract of Gingyo-san (8.9—12.5 mg/0.25 ml/mouse×3 per day) significantly reduced fever production and suppressed the rise in IL-1α production to the level in uninfected mice. No apparent toxicity by Gingyo-san was observed in infected mice. When the hot water-extract of each 10 of the crude components of Gingyo-san (an unknown amount extracted from 6.25 mg/0.25 ml/mouse×3 per day for Saigae Tataricae Cornu and 3.5 mg/0.25 ml/mouse×3 per day for the other 9) was orally administered to infected mice, 6 showed significant antipyretic activity. Of these 6, Saigae Tataricae Cornu significantly suppressed the rise in IL-1α production to the basal level while the other 5 did not affect serum IL-1α. Thus, of the 10 crude components of Gingyo-san, Saigae Tataricae Cornu simultaneously exhibited antipyretic and IL-1α-regulatory activities. The novel antipyretic action of Gingyo-san may be mainly mediated by Saigae Tataricae Cornu which regulates the elevated serum IL-1α level produced by influenza infection.

Key words crude drug; antipyretic activity; interleukin-1α; Saigae Tataricae Cornu; Gingyo-san

Fever is one of the major symptoms in the acute phase of influenza but the precise mechanism of fever production is not clearly understood. In order to analyze the fever production caused by infection, we previously selected a mouse strain which is the most susceptible to fibrile responses against interferon (IFN) among 7 strains, and developed a fever induction model due to influenza virus infection using this strain. In this model, we have evaluated the relationship involving IFN activity, interleukin (IL)-1α production and cyclooxygenase (COX) activity for fever production and demonstrated a fever cascade as follows: influenza virus infection, IFN production, IL-1α production, elevated COX activity and prostaglandin (PG)E_2 production (COX–PGE_2), fever induction.[1)] Based on this cascade, we demonstrated that Kakkon-to reduced fever production by suppressing the rise in IL-1α production subsequent to IFN production in influenza virus-infected mice.[2)] Further, we have isolated and characterized compounds with such antipyretic activity from a crude component of Kakkon-to.[3)] Cinnamyl derivatives and related compounds have been found to play an important role in the novel antipyretic action of Kakkon-to and the mode of their antipyretic action was confirmed in influenza virus-infected mice.[3)] However, the antipyretic activity of these compounds was not observed in mice given IL-1α injections in contrast to aspirin, indicating that they interfere with the production of IL-1α by influenza virus infection.[3)] Such novel antipyretic action differs from that of aspirin which shows antipyretic activity by inhibiting COX activity, and so suppressing the production of PGE_2 and fever.[1—3)] Thus, this model is suitable for assessing the mechanism of drugs with antipyretic activity in influenza virus infection.

Gingyo-san (GS, Yinqino-aan), a traditional medicine, is the most popular medicine for the common cold, especially influenza infection in China and has been used since the time of ancient China. Its major benefit is known to be its antipyretic activity.[4)] Thus, in the model of murine fever induced by influenza virus infection, we have analyzed the antipyretic action of GS and its 10 crude components. The relationship between defervescence and the suppression of IL-1α production by GS was evaluated. GS exhibited antipyretic activity and reduced IL-1α production in influenza virus-infected mice. Of the 10 crude components of GS, Saigae Tataricae Cornu simultaneously showed antipyretic and IL-1α-regulatory activities and may mainly contribute to the novel antipyretic action of GS by regulating the serum IL-1α level elevated by influenza infection. Thus, the efficacy of GS, as a traditional antipyretic medicine, in influenza has been verified.

Experimental

Preparation of Hot Water (HW)-Extracts of GS and Its Crude Components GS is composed of 10 crude drugs, Forsythiae Fructus, Menthae Herba, Schizonepetae Spica, Sojae Semen Preparatum, Glycyrrhizae Radix, Saigae Tataricae Cornu, Lonicerae Flos, Platycodi Radix, Lophatheri Herba and Arctii Fructus and was prepared as the Tenshin-kanbo-hen according to the Chui-syoho-kaisetsu, 1982.[4)] HW-extracts were prepared from GS (GS-1 and -2) containing different lots of 10 crude drugs as its components. The dried materials of 10 crude drugs (4.26 g Forsythiae Fructus, 2.556 g Menthae Herba, 1.704 g Schizonepetae Spica, 2.136 g Sojae Semen Preparatum, 2.556 g Glycyrrhizae Radix, 0.132 g Saigae Tataricae Cornu, 4.26 g Lonicerae Flos, 2.556 g Platycodi Radix, 1.704 g Lophatheri Herba, 2.136 g Arctii Fructus) were boiled under reflux in a 10-fold volume of distilled water for 80 min. The aqueous extract was filtered through 4 sheets of gauze, concentrated under reduced pressure at 50 °C, and lyophilized.[5)] The lyophilized materials were resuspended in distilled water (35.6 mg/ml). The suspension was warmed at 40—50 °C for 10 min and given orally to mice.[2,6)]

Tablets of GS (GS-3, Seisei-gingyo-gedoku-hen, China) are commercially

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available in China and mainly contain the 10 crude drugs in dried form. However, some details of the contents are unknown. The tablets were powdered and suspended in distilled water at 50 mg/ml and warmed as described above. Since Kakkon-to, a traditional herbal medicine, has been previously shown to exhibit antipyretic activity in influenza virus-infected mice,[2] this HW-extract was used as a positive control to evaluate the antipyretic activity of the HW-extracts of traditional medicines. This herbal medicine was supplied from Kanebo, Ltd., Japan.

For the preparation of HW-extracts, 20—50 g of each crude drug, except for Saigae Tataricae Cornu, was boiled under reflux in a 10-fold volume of distilled water for 60 min. The aqueous extract was filtered and lyophilized. The lyophilized materials were then suspended in distilled water at 14 mg/ml for oral administration to mice as described above. We could not obtain enough dried materials from Saigae Tataricae Cornu to prepare a HW-extract suspension for oral administration to mice. Therefore, Saigae Tataricae Cornu was suspended in distilled water at 50 mg/ml and boiled for 15 min. Then the suspension was centrifuged at 3000 rpm for 10 min. The supernatant was diluted with an equal volume of distilled water and directly used for oral administration to mice without lyophilization.

Mouse Influenza Virus Infection Model Mouse-adapted influenza virus [A/PR/8/34 (H1N1)] was prepared from the lungs of infected mice as described previously.[7] Female DBA/2 Cr mice (6-week-old, 17—19 g, Sankyo Labo Service Co., Ltd., Japan) were intranasally infected or mock-infected with 2000—3000 plaque forming units of influenza virus under ether anesthesia. HW-extracts of GS and Kakkon-to or water were administered orally by gavage to the mice three times daily, at approximately 8 h intervals, for 4 d starting a day before infection, at doses (8.9 mg/0.25 ml/mouse for GS-1 and -2, 12.5 mg/0.25 ml/mouse for GS-3 and 5 mg/0.25 ml/mouse for Kakkon-to) corresponding to the conventional doses of dried traditional medicines used for humans. Five to 10 mice were used in each group. The rectal temperature was monitored by a thermometer (Sato Keiryoki MFG, Co., Ltd. Ltd, Japan) immediately before infection and at 27, 33 and 44 h after infection, at which time fever, as previously shown,[1,2] is produced in the murine influenza infection model. After the measurement of rectal temperature, sera were prepared from 4 to 5 mice in each group under ether anesthesia. IL-1α concentrations in sera were determined by enzyme-linked immunosorbent assay (ELISA) using ELISA kits for mouse IL-1α (Genzyme, U.S.A.) according to the manufacturer's instructions.[5] To determine the survival rate, the infected mice were given GS as described above and fed without its administration at 2 d postinfection.

Ten crude components of GS were orally administered as described above. The doses used for mice were the amount extracted from 6.25 mg/0.25 ml/mouse for Saigae Tataricae Cornu, as described above, and 3.5 mg/0.25 ml/mouse for the other 9 crude drugs to evaluate their possible antipyretic activity. In the case of Saigae Tataricae Cornu, 6.25 mg/0.25 ml/mouse of Saigae Tataricae Cornu was used as the dose corresponding to a dose of HW-extract of GS, although the exact amount was uncertain. The doses of the other 9 corresponded to the doses for mice at which the crude components of Kakkon-to exhibited antipyretic activity as described previously.[3] The rectal temperature of each mouse was monitored and IL-1α concentrations in sera were determined as described above.

Statistical Analysis Student's *t*-test was used to evaluate any statistically significant differences between the two groups in terms of rectal temperature and IL-1α concentration in sera. Statistical differences in survival rate were evaluated each day after infection using Fisher's exact test. A p value of less than 0.05 was defined as statistically significant.

Results

Effects of GS on Fever Production The HW-extracts of GS were examined for their antipyretic activity in a murine influenza virus infection model. As shown in Fig. 1, fever developed 33 to 44 h after influenza infection. However, oral administration of GS significantly reduced fever production 33 to 44 h after infection compared with infected mice given only water (Fig. 1). In this murine infection model, Kakkon-to showed antipyretic activity as described previously.[2] In this experiment (Fig. 2), Kakkon-to was used as a positive control and reduced fever production significantly. Thus, our murine infection model was confirmed to be valid. Such suppression of fever production was also observed in infected

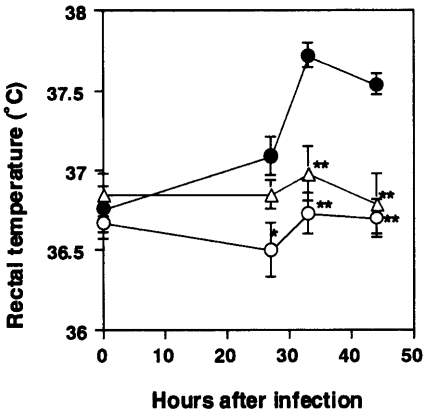

Fig. 1. Changes in Rectal Temperature in Influenza Virus-Infected Mice

Ten mice in each group were intranasally infected (●) or mock-infected (uninfected, ○) with influenza virus. GS (△) or water (● and ○) was administered orally to the mice and the rectal temperature was monitored at 0, 27, 33 and 44 h after infection as described in the text. Vertical bars indicate standard error. * $p<0.05$ *vs.* infected mice given water alone using Student's *t*-test. ** $p<0.01$ *vs.* infected mice given water alone using Student's *t*-test.

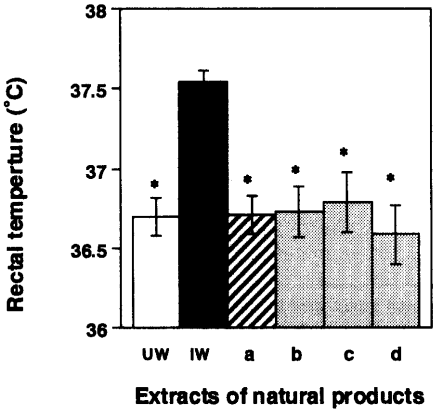

Fig. 2. Effects of 3 Kinds of GS [GS-1 (b), 2 (c) and 3 (d)] and Kakkon-to (a) on Fever Production in Influenza Virus-Infected Mice

Ten mice in each group were intranasally infected or mock-infected (uninfected) with influenza virus. GS or water was administered orally to the mice and the rectal temperature was monitored at 44 h after infection as described in the text. Vertical bars indicate standard error. UW and IW represent uninfected and infected mice, respectively, given water alone. * $p<0.01$ *vs.* infected mice given water alone using Student's *t*-test.

mice given three different kinds of GS (Fig. 2). There was no difference in antipyretic activity among the GS lots. Oral administration of GS did not affect the survival rate of infected mice and infected mice began to die starting 4 d postinfection as shown in Fig. 3. No apparent toxicity by GS was observed for 3 d after infection. GS exhibited antipyretic activity in influenza virus-infected mice.

Effects of GS on IL-1α Production Defervescence has been shown to be caused by suppressing IFN-induced IL-1α production in a murine intranasal influenza infection model.[2] In order to evaluate the relationship between defervescence and the suppression of IL-1α production by GS, the level of IL-1α production in serum was examined in influenza virus-infected mice. Compared with infected mice given only water, GS, as well as Kakkon-to, suppressed the rise of IL-1α production significantly when fever developed 44 h after infection (Table 1). GS was effective in reducing the rise in IL-1α production in influenza virus-infected mice. Thus, GS simultaneously exhibited antipyretic activity and regulated IL-1α production in infected mice.

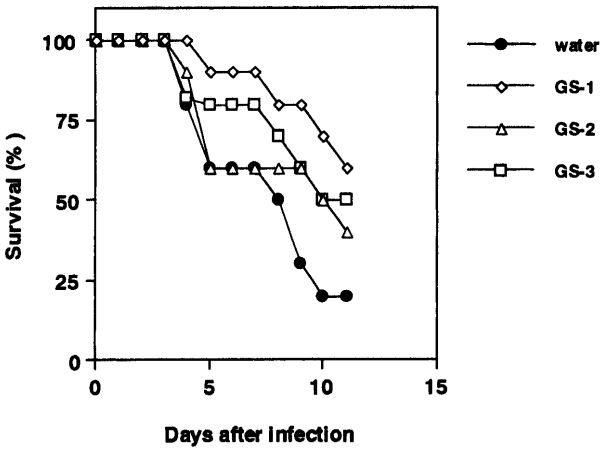

Fig. 3. Survival Time of Influenza Virus-Infected Mice

Mice were intranasally infected with influenza virus. GS-1 (◇), 2 (△) and 3 (□) and water (●) were orally administered as described in the text. Ten mice were used in each group.

Table 1. Effects of GS on IL-1α Production in Serum of Influenza Virus-Infected Mice

Mice	Treatment	IL-1α (pg/ml)
Uninfected	Water	146.5±17.1 (0.005)
Infected	Water	187.5±16.3
	Kakkon-to	154.0±18.6 (0.016)
	GS-1	145.0±8.3 (0.001)
	GS-2	150.5±16.3 (0.007)
	GS-3	130.0±9.3 (0.001)

Five mice in each group were intranasally infected or mock infected (uninfected) with influenza virus. Three kinds of GS (GS-1, 2, 3) or water alone was administered orally to the mice and IL-1α concentrations (the mean±S.D.) in sera were determined at 44 h after infection by ELISA kits as described in the text. Parentheses indicate statistical significance (p values) *vs.* infected mice given water alone using Student's t-test.

Effects of 10 Crude Drugs on Fever and IL-1α Production Each HW-extract of the 10 crude drugs involved in GS was examined for their antipyretic activity and ability to regulate IL-1α production in a murine influenza virus infection model. Among the 10 crude drugs, Forsythiae Fructus, Menthae Herba, Schizonepetae Spica, Sojae Semen Preparatum, Glycyrrhizae Radix and Saigae Tataricae Cornu significantly suppressed fever production compared with infected mice given only water (Fig. 4). Among the 6, Saigae Tataricae Cornu showed the strongest antipyretic activity (Fig. 4). Only Saigae Tataricae Cornu significantly suppressed the rise in IL-1α production compared with infected mice given only water (Table 2). Thus, among the 10 crude drugs, Saigae Tataricae Cornu was effective in reducing both fever production and the rise in IL-1α production in influenza virus-infected mice.

Discussion

Oral administration of GS resulted in antipyretic activity in influenza virus-infected mice (Figs. 1, 2). GS is a common and traditional medicine used to treat the common cold and influenza in China and it produces defervescence.[4,5] In this study, the doses of GS for mice were calculated from the conventional doses used for humans, based on body surface area.[8,9] These doses did not affect the mortality of infected mice (Fig. 3), suggesting that the toxicity of GS was low. Thus, its antipyretic activity, recognized since ancient time,

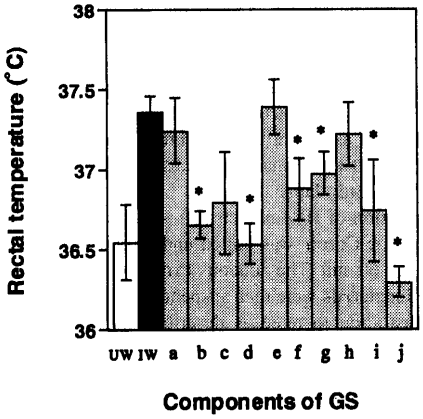

Fig. 4. Effects of the 10 Crude Drugs on Fever Production in Influenza Virus Infected Mice

Ten mice in each group were intranasally infected or mock-infected (uninfected) with influenza virus. GS or water was administered orally to the mice and the rectal temperature was monitored at 44 h after infection as described in the text. UW and IW represent uninfected and infected mice, respectively, given water alone. Infected mice were given Forsythiae Fructus (a), Menthae Herba (b), Schizonepetae Spica (c), Sojae Semen Preparatum (d), Arctii Fructus (e), Glycyrrhizae Radix (f), Lonicerae Flos (g), Platycodi Radix (h), Lophatheri Herba (i) and Saigae Tataricae Cornu (j). Vertical bars indicate standard error. * $p<0.01$ *vs.* infected mice given water alone using Student's t-test.

Table 2. Effects of 10 Crude Drugs on IL-1α Production in Serum of Influenza Virus-Infected Mice

Mice	Treatment	IL-1α (pg/ml)
Uninfected	Water	75.5±22.0* (0.013)
Infected	Water	143.5±42.4
	Lonicerae Flos	133.0±8.9
	Forsythiae Fructus	114.0±20.5*
	Platycodi Radix	117.5±19.0
	Menthae Herba	107.0±17.2*
	Lophatheri Herba	133.5±9.5
	Schizonepetae Spica	120.5±9.9*
	Sojae Semen Preparatum	116.5±19.8*
	Arctii Fructus	122.5±19.5
	Glycyrrhizae Radix	117.5±14.3*
	Saigae Tataricae Cornu	96.5±29.8* (0.007)

Five mice in each group were intranasally infected or mock infected (uninfected) with influenza virus. Each of the 10 components of GS or water alone was administered orally to the mice and IL-1α concentrations (the mean±S.D.) in sera were determined at 44 h after infection by ELISA kits as described in the text. Parentheses indicate statistical significance (p values) *vs.* infected mice given water alone using Student's t-test. Asterisks indicate those crude drugs which reduced fever production in infected mice as shown in Fig. 4.

was confirmed in our murine infection model.

Among the 10 kinds of crude drugs involved in GS, 6 showed antipyretic activity in infected mice (Fig. 4). The antipyretic activity of GS was shown to be mainly due to these 6 crude drugs. GS exhibited IL-1α-regulatory activity as well as antipyretic activity in influenza virus-infected mice (Table 1). However, among the 6 crude drugs with antipyretic activity, only Saigae Tataricae Cornu significantly exhibited IL-1α-regulatory activity (Table 2). Thus, Saigae Tataricae Cornu may be mainly responsible for the IL-1α-regulatory activity of GS and the other 5 crude drugs may have a different mode of antipyretic action from that of Saigae Tataricae Cornu.

Cinnamyl derivatives, as well as aspirin, has been shown to exhibit anti-inflammatory activity as well as antipyretic activity.[10—15] We have recently selected the antipyretic compounds, 7-hydroxycoumarin, 4-allylanisole, cinnamic acid

ethylester, acetic acid cinnamylester, 2′-hydroxyacetophenone and 2-hydroxycinnamic acid, from 48 cinnamyl derivatives and related compounds using a murine influenza virus infection model.[3] Of these cinnamyl derivatives and related compounds, 7-hydroxycoumarin, 4-allylanisole, cinnamic acid ethylester and acetic acid cinnamylester also exhibited IL-1α-regulatory activity and it was suggested that they had a different mode of antipyretic action from that of aspirin.[3] It is possible that the modes of biological action of these compounds may differ even although their action is the same. Cinnamyl derivatives and aspirin have been isolated and identified as components of plants.[3,4,16,17] Although Saigae Tataricae Cornu exhibited antipyretic activity (Fig. 4), this crude drug comes from animal horn. Thus, Saigae Tataricae Cornu may contain different compounds with antipyretic activity compared with cinnamyl derivatives and aspirin.

The antipyretic activity of Kakkon-to and its components (cinnamyl derivatives and related compounds) has been previously shown to correlate with IL-1α-regulatory activity in infected mice.[2,3] In influenza virus-infected mice, defervescence occurred by suppressing IFN-induced IL-1α production.[3] When GS antipyretic activity was observed, IL-1α-regulatory activity was also present (Fig. 2, Table 1). Also, Saigae Tataricae Cornu simultaneously exhibited both activities (Fig. 4, Table 2). The antipyretic activity of GS and Saigae Tataricae Cornu correlated with their IL-1α-regulatory activity. Thus, they may be effective in reducing fever production by suppressing IFN-induced IL-1α production in influenza. GS possibly exhibits its novel antipyretic action by regulating serum IL-1α levels elevated by influenza infection and its novel action may be mainly mediated by Saigae Tataricae Cornu.

GS, as well as Kakkon-to, showed novel antipyretic activity possibly by suppressing the production of IL-1α caused by influenza infection. GS is very widely used for the treatment of influenza in China while Kakkon-to is mainly used in Japan. It is interesting that these traditional medicines, showing similar antipyretic action, are used in different countries to treat influenza.

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