Kakkon-to suppressed interleukin-1α production responsive to interferon and alleviated influenza infection in mice

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Abstract

We evaluated the therapeutic efficacy of Kakkon-to in an intranasal influenza infection model in mice. Oral administration of Kakkon-to caused the early recovery of body weight in infected mice ($p<0.05$ or 0.01 by the repeated measure analysis of variance), retarded the development of pneumonia ($p<0.05$ by the Student's t-test) and decreased mortality of infected mice ($p<0.05$ by the Fisher's exact test) as compared with water-administered groups. This treatment also suppressed fever 1 to 2 days after infection ($p<0.05$ by the Student's t-test). Since Kakkon-to treatment did not affect the growth of influenza virus in the lungs, we examined its effects on the production of cytokines as immune mediators against influenza infection. Kakkon-to treatment did not affect interferon (IFN) activity and the levels of interleukin (IL)-2, tumor necrosis factor-α and IFN-γ in serum after infection as compared with water administration. However, Kakkon-to significantly suppressed the rise of IL-1 α level in serum and the bronchoalveolar lavage fluid of lungs compared with untreated mice ($p<0.01$ or 0.05 by the Student's t-test). When fever abated in infected mice treated with Kakkon-to, IL-1 α level also decreased in serum ($p<0.05$ by the Student's t-test) and was maintained at the level of uninfected mice. Thus, the alleviation of pneumonia and antipyretic action by Kakkon-to were possibly based on the suppression of IL-1 α production induced by IFN in influenza. Kakkon-to was confirmed to have advantages in making pneumonia milder and reducing fever in infected mice.

Key words Kakkon-to, influenza infection, pneumonia, fever, cytokine, interleukin-1 α, medicinal herb.

Abbreviations ANOVA, prostaglandin analysis of variance; COX, cyclooxygenase; COX-PGE_2, cyclooxygenase activity and prostaglandin E_2 production; ELISA, enzyme-linked immunosorbent assay; HSV, herpes simplex virus; IU, international unit; IL, interleukin; IFN, interferon; MDCK, Madin-Darby canine kidney; MEM, Eagle's minimum essential medium; PBS, phosphate-buffered saline; PG, prostaglandin; TNF, tumor necrosis factor; VSV, Vesicular stomatitis virus.

Introduction

Kakkon-to, a traditional herbal medicine, is composed of 7 medicinal herbs, *Pueraria pseudo-hirsuta* TANG et WANG (Radix), *Ephedra sinica* STAPF (Cortex), *Zizyphus jujuba* MILL. (Fruit), *Cinnamomum cassia* BLUME (Cortex), *Paeonia lactiflora* PALL (Radix), *Glycyrrhiza uralensis* FISCH. (Radix), and *Zingiber officinale* ROSC. (Rhizome).[1,2] This herbal medicine has been used for the treatment of influenza infection and the common cold since ancient times in China and more than 20 million doses are prescribed annually in Japan. Although the antipyretic action of Kakkon-to has been recognized as a major benefit in the treatment,[1] its pharmacological and biochemical

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bases have not been clearly understood for its antipyretic action.

We have been certifying the efficacy of traditional medicines in viral infection using experimental animal models.[2-5] In our previous study, Kakkon-to did not inhibit the growth of herpes simplex virus (HSV) *in vitro* but it exhibited therapeutic efficacy in HSV-infected mice and reduced their mortality.[2] The efficacy of Kakkon-to was demonstrated to result from the augmentation of delayed type hypersensitivity to HSV.[2] Thus, a murine infection model has been useful in analyzing the mode of action of traditional medicines *in vivo*.

We utilized an intranasal influenza virus infection model in mice to evaluate the therapeutic efficacy of Kakkon-to. This murine model is a good model for characterizing the development of typical pneumonia with pathologic changes similar to those in humans.[6,11] Also, this model using DBA/2 Cr mice has been shown to be the most suitable model for analyzing the mechanism of fever production among 7 mouse strains.[6] We have demonstrated the cascade of fever production in influenza infection using the DBA/2 Cr mouse model as follows: influenza virus infection, elevated interferon (IFN) activity, interleukin (IL)-1 α production, elevated cyclooxygenase (COX) activity and prostaglandin (PG)E_2 production (COX-PGE_2), fever induction.[6] In this study, we showed that Kakkon-to treatment alleviated pneumonia and reduced fever in influenza infection. The alleviation of pneumonia and novel antipyretic action by Kakkon-to were suggested to originate in the suppression of responsive IL-1α production subsequent to IFN production after infection.

Materials and Methods

Cells and viruses : Madin-Darby canine kidney (MDCK) cells and mouse L 929 cells were grown and maintained in Eagle's minimum essential medium (MEM) supplemented with 5 % and 2 % heat-inactivated calf serum, respectively. Mouse-adapted influenza virus [A/PR/8/34 (H1N1)] was prepared from the lungs of infected mice as described previously.[6] Vesicular stomatitis virus (VSV, New Jersey strain) was provided from the National Institute of Animal Health, Japan and used for the IFN assay.[2]

Preparation of Kakkon-to : Kakkon-to was supplied from Tsumura & Co., Ltd., Japan as the lyophilized powder. The powder was suspended in distilled water at 20 mg/ml. The suspension was warmed up at 40°C for 15 min and used for oral administration to mice. The administration dose (750 mg/kg/day) of Kakkon-to for mice was determined from that for human use.[2,5] Three lots of Kakkon-to were used in this study.

Mouse influenza virus infection model : Female ICR or DBA/2 Cr mice (5 or 6-week-old, respectively, 18-20 g, Sankyo Labo Service Co., Ltd., Japan) were intranasally infected or mock-infected with 800-1,000 plaque forming units of influenza virus under ether anesthesia. Kakkon-to or water was administered orally to the mice three times daily (approximately 8 hr interval) for 7 days starting a day before infection. The development of consolidation of lungs was observed and scored as described previously.[6,12] The lungs were also examined histopathologically in untreated and Kakkon-to-treated mice as described previously.[2] Body weight of each mouse was monitored every morning after infection and the rectal temperature was also done by a thermometer (Sato Keiryoki MFG, Co., Ltd, Japan). To determine their mortality, the infected mice were fed and observed for at least 20 days after infection.

Virus yield in lungs : Virus yields in lungs were examined in influenza virus-infected mice treated with or without Kakkon-to. The lungs of infected mice were removed under ether anesthesia on days 2, 4, 6, 8 and 10 after infection. The lung was homogenized in phosphate-buffered saline (PBS) at a concentration of 10 % (w/v) followed by a centrifugation at 3,000 rpm for 15 min. Virus titer of the supernatant was determined by the plaque assay using MDCK cells as described previously.[13]

Determination of serum IFN activity : IFN activity in serum was determined by the plaque reduction assay of VSV in mouse L929 cells as reported previously.[2,6] Serum IFN level was expressed as the IFN international unit (IU) by comparison with standard recombinant murine IFN-α (10^9 IU/mg, Gibco BRL).

Determination of cytokines in serum or bronchoalveolar lavage fluid : The levels of cytokines in the

bronchoalveolar lavage fluid of lungs or serum were examined by the enzyme-linked immunosorbent assay (ELISA). The bronchoalveolar lavage fluid was obtained by instilling 1 ml of MEM into lungs and aspirating it from the trachea of mice using tracheal cannula as described previously.[6] Cytokine levels in the bronchoalveolar lavage fluid or serum were determined using ELISA kits for mouse IL-1 α, IL-2, tumor necrosis factor (TNF)-α, and IFN-γ (Genzyme, USA) according to the manufacturer's instructions.

Murine model for fever production: The effect of Kakkon-to on fever production was examined mainly in DBA/2 Cr mice.[6] Infection and Kakkon-to treatment were performed as described above. For comparing anti-pyretic actions of Kakkon-to and aspirin, aspirin (80 mg/kg/day) was administered orally to mice immediately and approximately at 8 hr interval after infection. Rectal temperatures were monitored periodically as indicated in the text.

Statistical analysis: The Student's t-test was used to evaluate the statistical significance of differences between two groups in rectal temperatures, the concentrations of cytokines in bronchoalveolar lavage fluid and serum, and IFN activities. The repeated measure analysis of variance (ANOVA) was used to analyze the interaction between Kakkon-to and water in the rise of body weight of infected and uninfected mice. Statistical differences in the mortality were evaluated using the Fisher's exact test. A p value of less than 0.05 was statistically defined as significant.

Results

Therapeutic efficacy of Kakkon-to in mice

Therapeutic efficacy of Kakkon-to was evaluated in an intranasal influenza virus infection model in mice. When ICR mice were infected with influenza virus, the body weight decreased markedly later than 2 days after infection (Fig. 1A) and the consolidation of lungs was obviously observed on day 4 postinfection.[6] However, Kakkon-to treatment delayed the decrease of body weight and caused the early recovery of body weight (Fig. 1A, $p < 0.01$ by the repeated measure ANOVA). The development of consolidation was also retarded in Kakkon-to-treated mice (data not shown). Histopathological analysis of lungs showed the significant retardation in the development of pneumonia in Kakkon-to treated mice (Fig. 2A, $p < 0.05$ by the Student's t-test). As shown in Fig. 3 as one of the representative results, Kakkon-to treatment

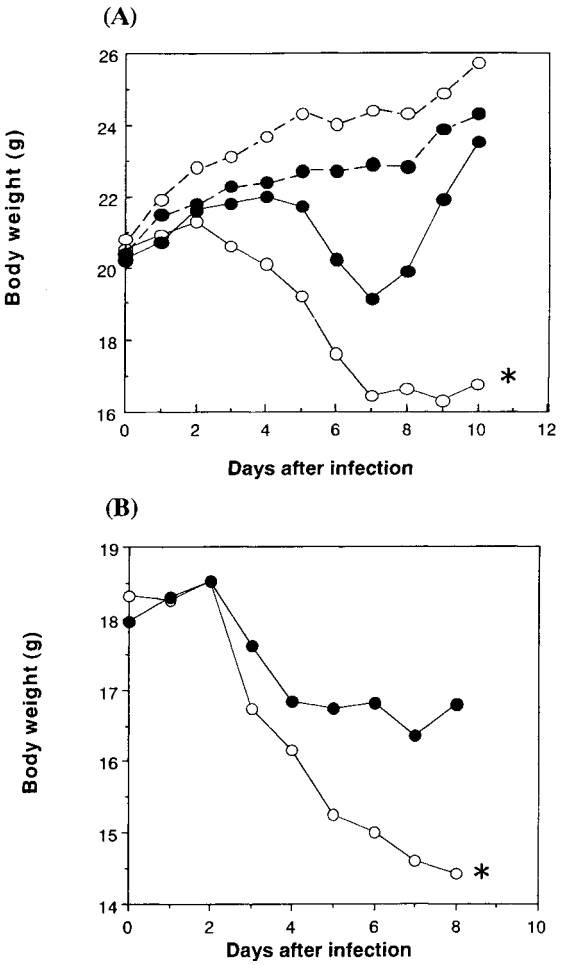

Fig. 1 Effects of Kakkon-to on the body weight of mice. ICR (A) mice were mock-infected (broken lines) or infected (solid lines) intranasally with influenza virus. Kakkon-to (closed circles) and water (open circles) were orally administered as described in the text and the body weight was measured daily. Five to 10 mice were used in each group. Asterisk indicates significant difference in infected mice with Kakkon-to-administration and uninfected mice with water-and Kakkon-to-administration for 1 to 10 days postinfection, $p < 0.01$ by the repeated measure ANOVA.

DBA/2 Cr (B) mice were infected intranasally with influenza virus. Kakkon-to (closed circles) and water (open circles) were orally administered as described in the text and the body weight was measured daily. Five to 10 mice were used in each group. Asterisk indicates significant difference from infected mice with Kakkon-to-administration for 3 to 8 days postinfection, $p < 0.05$ by the repeated measure ANOVA.

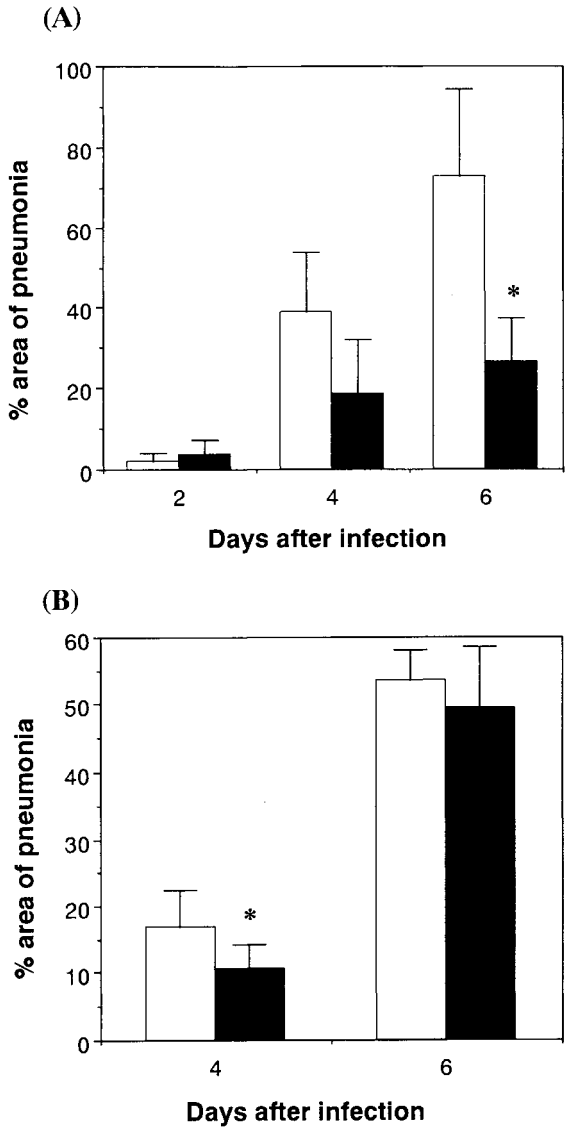

Fig. 2 Effect of Kakkon-to on the development of pneumonia in mice infected with influenza virus. ICR(A) or DBA/2 Cr (B) mice were infected intranasally with influenza virus, and Kakkon-to and water were orally administered as described in the text. The lungs were removed from 3 to 4 mice in each group on days 2, 4 and 6 after infection. Open and closed columns show water- and Kakkon-to-administered groups, respectively. Asterisk indicates significant difference from water-administered group, $p<0.05$ by the Student's t-test.

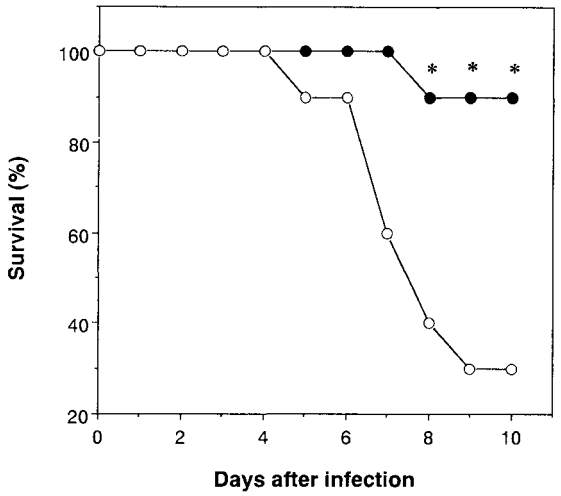

Fig. 3 Survival time of influenza virus-infected mice. ICR mice were intranasally infected with influenza virus. Kakkon-to (closed circles) and water (open circles) were orally administered as described in the text. Ten mice were used in each group. Asterisk indicates significant difference from water-administered group, $p<0.05$ by the Fisher's exact test.

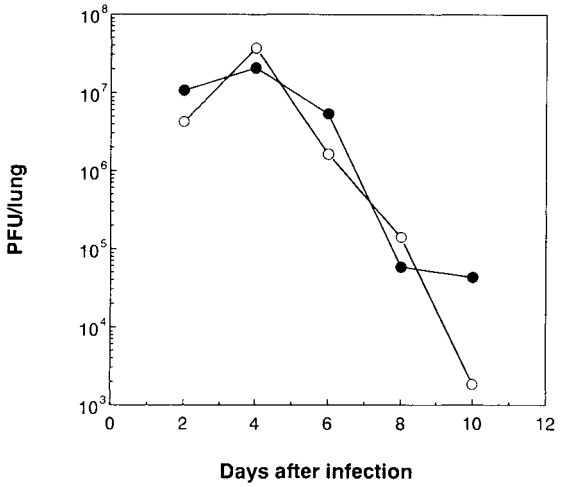

Fig. 4 Effect of Kakkon-to on the growth of influenza virus in lungs. ICR mice were infected intranasally with influenza virus, and Kakkon-to (closed circles) and water (open circles) were orally administered as described in the text. The lungs were removed from 3 mice in each group on days 2, 4, 6, 8 and 10 after infection and homogenization. Virus titer in the supernatant of the homogenate was determined by the plaque assay and the mean values of 3 lungs were plotted.

reduced the mortality of infected mice ($p<0.05$ by the Fisher's exact test). The early recovery of body weight, the alleviation of pneumonia and the reduction in mortality by Kakkon-to were confirmed by repeating experiments using other preparations of Kakkon-to. However, Kakkon-to treatment did not affect virus yields in the lungs of infected mice (Fig. 4).

In DBA/2 Cr mice infected with influenza virus, the pathologic changes of lungs became evident micro-

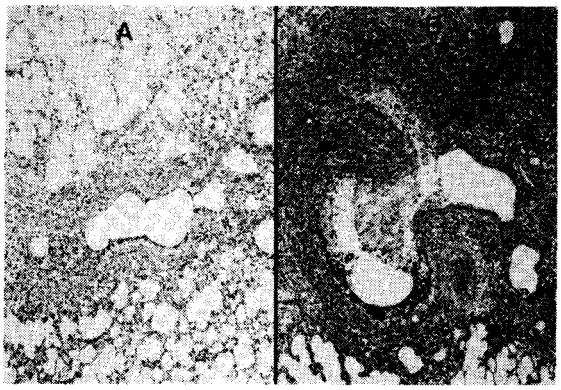

Fig. 5 Histopathological analysis of lungs in influenza virus-infected mice. The lungs of Kakkon-to-treated (A) and untreated (B) mice were removed on day 4 after infection. The resected lung was fixed in 10 % formalin solution, dehydrated and embedded in paraffin. Four-micrometer thick sections were cut and stained with hematoxylin and eosin.

scopically on day 4 after infection and progressed later. The epithelium of bronchi and bronchioles showed infiltration of inflammatory cells and necrosis, and was overlaid by mucopurulent materials. These pathologic changes of lungs were milder in Kakkon-to-treated mice than in water-administered mice (Fig. 2B, $p<0.05$ by the Student's t-test and Fig. 5). The delay of loss of body weight by Kakkon-to treatment was also confirmed in the infected DBA/2 Cr mice (Fig. 1B, $p<0.05$ by the repeated measure ANOVA). Therefore, Kakkon-to treatment was confirmed to exhibit therapeutic efficacy in alleviating pneumonia in an influenza infection model in mice.

Effect of Kakkon-to on production of cytokines in infected mice

Effect of Kakkon-to on the production of cytokines including IFN as an immune mediator was examined in ICR mice infected with influenza virus. IFN activity increased in serum on day 2 after infection as described previously.[6] At this time, Kakkon-to treatment did not affect the increase of IFN activity (Table I). Among cytokines examined, only IL-1 α level increased in both bronchoalveolar lavage fluid of lungs and serum mainly 2 days after infection as described previously.[6] However, Kakkon-to treatment reduced IL-1 α level in the bronchoalveolar lavage fluid and serum on day 2 after infection significantly and preserved its level at that in uninfected mice (Table II, $p<0.05$ by the Student's t-test), whereas the levels of other cytokines (IL-2, TNF-α and IFN-γ) examined were not affected by Kakkon-to treatment (data not shown). Therefore, Kakkon-to treatment permitted the increase of IFN activity but suppressed IL-1 α production responsive to IFN.[14]

Table I IFN activity in sera obtained from influenza virus-infected mice*

Treatment	IFN titer, IU[a]	
	Uninfected	Infected
Water	53.3± 3.8	172.6±63.6[b]
Kakkon-to	29.2±22.3	127.9±28.4[c]

*IFN activity in serum was examined by the plaque reduction assay of VSV in mouse L929 cells. Infected-and uninfected mice were administered with water or Kakkon-to (750 mg/kg/day) and sera taken on day 2 after infection were examined for IFN activity as described in the text. Four to 5 mice were used in each group.
[a]Serum IFN levels were expressed as the international unit.
[b]$p<0.05$ vs. untreated mice with water administration.
[c]$p<0.01$ vs. untreated mice with Kakkon-to administration.

Effect of Kakkon-to on fever production

Effect of Kakkon-to on fever production was examined in mice infected with influenza virus. In this

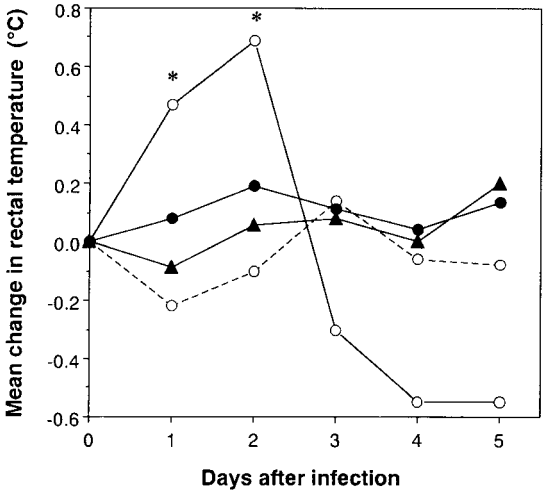

Fig. 6 Effects of Kakkon-to on the mean rectal temperatures of DBA/2 Cr mice. The mice were mock-infected (broken lines) or infected (solid lines) intranasally with influenza virus. Kakkon-to (closed circles), aspirin (closed triangle) or water (open circles) was orally administered as described in the text and the rectal temperature was measured every morning. Five to 7 mice were used in each group. Asterisk indicates significant defference from water-administered, aspirin-administered and mock-infected groups, $p<0.05$ by the Student's t-test.

Table II Amounts of IL-1 α in the bronchoalveolar lavage fluid of lungs and serum from mice treated with or without Kakkon-to.*

Expt. No.	Days after infection	IL-1 α (mean ±S.D.), pg/ml			
		Unifected mice		Infected mice	
		Water-treated	Kakkon-to-treated	Water-treated	Kakkon-to-treated
1. Serum (n=2 or 3)[a]					
	2	40.4±11.6[b]	42.8±22.8	122.4±18.8[b]	62.4±25.2[d]
	4	16.4±10.8[b]	22.0±7.2	46.4±5.2[b]	45.2±29.6
	6	24.0[b,c]	8.0[c]	30.8±16.4[b]	54.4±8.0
Lavage (n=3)					
	2	7.6±13.0[b]	4.8±3.0	65.2±23.6[b]	9.2±15.8[d]
	4	6.4±5.8[b]	0.8±1.4	45.6±40.4[b]	10.8±4.8
	6	0.0±0.0[b]	0.2±0.2	16.0±14.0[b]	6.0±10.4
2. Serum (n=3)					
	2	13.3±23.1	31.0±37.7	191.3±91.0	26.0±22.5[d]
	4			26.7±30.6	0.0±0.0
	6			31.0±37.7	26.7±46.2
Lavage (n=3)					
	2			32.6±11.8	4.6±7.9[d]
	4			22.8±20.2	5.4±2.4
	6			8.0±7.0	3.0±5.2
3. Serum (n=7 to 10)					
	2			80.2±39.4	44.8±14.2[d]
Lavage (n=2)					
	2	13.0[b,c]	5.0[c]	152.6[b,c]	64.1[c]
	4		5.6[c]	62.0[b,c]	80.6[c]
	6		5.0[c]	39.6[b,c]	15.6[c]
4. Serum (n=10)					
	2			139.2±28.8	87.3±34.8[e]

*IL-1 α levels in the bronchoalveolar lavage fluid of lungs and serum prepared from ICR mice were compared in Kakkon-to (750 mg/kg/day)- and untreated mice as described in the text.
[a]Parentheses indicate the number of mice used in each group.
[b]Values were cited from ref. 6.
[c]Mean of 2 samples.
[d]$p<0.05$ vs. infected mice with water administration.
[e]$p<0.01$ vs. infected mice with water administration.

experiment, we mainly used DBA/2 Cr mice for the analysis of fever production, because the elevation of temperature in DBA/2 Cr strain was the most prominent among 7 mouse strains examined as described previously.[6] Fever developed within 1 to 2 days after influenza infection (Fig. 6, $p<0.05$ by the Student's t-test). Such mode of fever was also observed in infected ICR mice. When fever was prominently produced on day 2 after infection (Fig. 6), IL-1 α levels rose significantly in sera of infected mice (Fig. 7, $p<0.05$ by the Student's t-test). However, Kakkon-to treatment as well as aspirin treatment reduced fever significantly (Fig. 6) and Kakkon-to treatment reduced IL-1 α concentration to the level of uninfected mice in the serum (Fig. 7). Thus fever was well correlated in the IL-1 α level in serum as reported previously[6] and the reduction of IL-1 α production responsive to IFN production by Kakkon-to probably caused the suppression of fever.

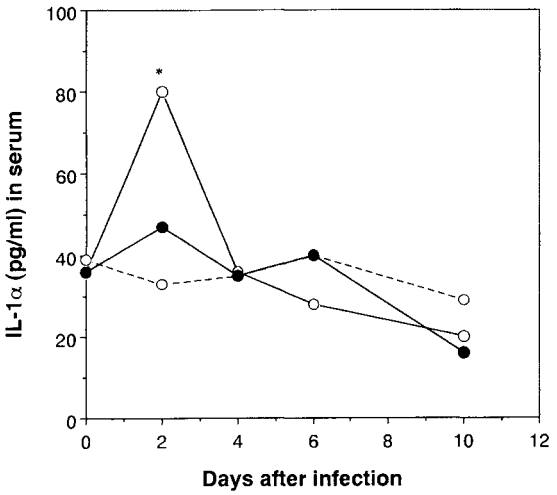

Fig. 7 Effects of Kakkon-to on the amount of IL-1 α in serum of mice. The mice were mock-infected (broken lines) or infected (solid lines) intranasally with influenza virus. Kakkon-to (closed circles) or water (open circles) was orally administered as described in the text. Whole blood was collected from 5-7 mice in each group on days 0, 2, 4, 6 and 10 after infection and serum was prepared. The concentrations of IL-1 α in serum were determined by the ELISA method as described in the text. Asterisk indicates significant difference from Kakkon-to-administered and mock-infected groups, $p<0.05$ by the Student's t-test.

Discussion

Kakkon-to has been historically used in the form of hot water-extract by oral administration and applied for the improvement of symptoms in acute phase of influenza virus infection. To evaluate its therapeutic efficacy on influenza virus infection, we used an intranasal influenza virus infection model in mice. This model is a lethal model with pneumonia,[7,8] and we have previously confirmed this in our infection models of ICR and DBA/2 Cr mice.[6] Kakkon-to was effective in reducing the severity of pneumonia, fever and the mortality of infected mice in our infection models. Thus the therapeutic efficacy of Kakkon-to on influenza virus infection was verified in this murine model.

Intranasal infection causes cellular infiltration in the respiratory tract of mice and the development of pneumonia results from pathological damage caused by an immune response against influenza virus infection.[9-11] In this study, pneumonia was milder in Kakkon-to-treated mice than in water-treated mice (Figs. 2 and 5). Virus yields were similar in the lungs of Kakkon-to- and water-treated mice (Fig. 4), indicating that Kakkon-to did not inhibit the growth of influenza virus in the lungs. Thus, the alleviation of pneumonia by Kakkon-to treatment could be due to the modification of an immunopathological response against influenza infection rather than the direct cytopathic effect of viral replication. Since IFN can induce IL-1 α production,[14] the augmentation of IFN activity in influenza infection would be able to increase IL-1 α production in a murine model[6] (Fig. 8). In an early stage of pneumonia, however, Kakkon-to treatment did not affect IFN activity but significantly reduced IL-1 α production to the level in untreated mice (Tables I and II), IL-1 α is produced from alveolar macrophages in the onset of the inflammatory response, and is produced only in early times postinfection in the lungs of infected mice.[9] Therefore the reduction of IL-1 α production suppressed cellular infiltration, and consequently may result in milder pneumonia in infected mice treated with Kakkon-to.

Influenza infection is known to produce fever as well as cytokines such as IFN, IL-1 and TNF-α,[9,19-22] and these cytokines are thought to produce fever and to be endogenous pyrogens.[14] As summarized in Fig. 8, we have previously shown that the fever production is induced in influenza virus-infected mice by the following cascade : elevated IFN activity, IL-1 α production, elevated COX-PGE_2.[6] Kakkon-to permitted the increase of IFN activity after infection (Table I) but suppressed IL-1 α production responsive to IFN (Fig. 7), resulting in reduction in fever. We have previously shown that aspirin, a representative antipyretic agent,[15-18] permits the increase of IFN activity and IL-1 α production but reduces COX-PGE_2 by inhibiting COX activity.[6] The inhibitory step of Kakkon-to was suggested to be prior to that of aspirin in the cascade (Fig. 8). Therefore, Kakkon-to has a different mode of antipyretic action from aspirin and one of the major antipyretic actions is due to the suppression of IL-1 α production responsive to IFN in infected mice.

Kakkon-to treatment was effective in retarding the development of pneumonia and prolonging the survival of mice. This treatment also alleviated fever

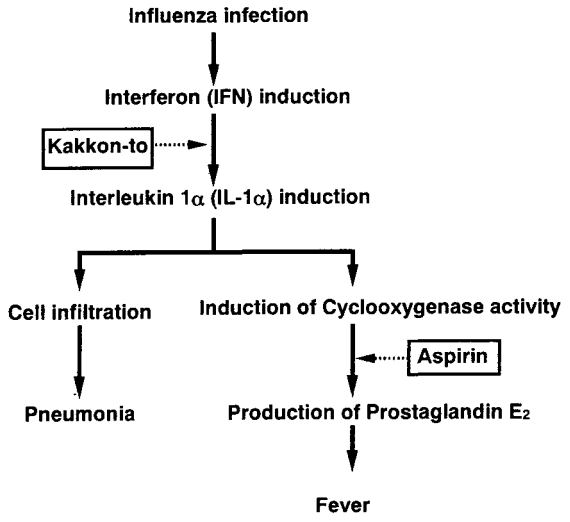

Fig. 8 Possible action of Kakkon-to on fever and pneumonia in influenza.

as a major symptom caused by influenza infection in the acute phase, and the novel antipyretic action of Kakkon-to was confirmed in febrile mice infected with influenza virus. These beneficial effects of Kakkon-to on pneumonia and fever correlated with the suppression of IL-1 α production responsive to IFN production in infected mice as described above. The suppression of responsive IL-1 α production by Kakkon-to may be one of the major actions leading the alleviation of pneumonia and defervescence in infected mice. IL-1α production possibly plays an important role in the defense system against influenza infection.[9] In spite of the reduction of IL-1 α production by Kakkon-to, pneumonia was alleviated without reducing virus yields in the lungs and the mortality of infected mice decreased. Kakkon-to treatment did not deteriorate the defense system even suppressing IL-1 α production in infected mice. Therefore IL-1 α production responsive to IFN may be an overreaction of the defense system in influenza infection. Kakkon-to was effective in limiting the overreaction in the pathogenesis of influenza infection possibly by suppressing responsive IL-1 α production.

Acknowledgments

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和文抄録

マウスの経鼻感染モデルを用いて，葛根湯のインフルエンザウイルス感染症における治療効果を検討した。葛根湯を経口投与したマウスでは，水投与群に比べ有意に体重減少が早期に回復し，肺炎の進展が遅延され，感染マウスの死亡率が低下した。また，葛根湯投与により，感染マウスの感染1，2日後の発熱が有意に抑制された。しかし，葛根湯投与によりマウス肺でのインフルエンザウイルス増殖は影響されなかった。このため，インフルエンザ感染における生体防御免疫因子と考えられるサイトカイン産生に及ぼす葛根湯の影響を検討した。葛根湯投与は，感染によるインターフェロン (IFN) 活性の上昇を抑制しなかった。また，感染後の血清中インターロイキン (IL)-2，腫瘍壊死因子-α，IFN-γ 濃度に影響しなかった。しかし，葛根湯投与感染マウスの肺胞洗浄液中，血清中で，IL-1 α 濃度の上昇が有意に抑制された。葛根湯投与により感染マウスの発熱が抑制されているときにも，感染による血清中 IL-1 α 濃度の上昇が有意に抑制され，非感染マウスの血清中 IL-1 α 濃度レベルに維持された。このため，葛根湯の肺炎軽症化作用，発熱抑制作用は，インフルエンザ感染で上昇した IFN 活性により誘導された IL-1 α産生の抑制に基づいている可能性が示唆された。これより，マウス感染系においても葛根湯のインフルエンザ感染による肺炎軽症化作用，発熱抑制作用が確認できた。

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