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Effect of Hochu-ekki-to (TJ-41), a Japanese herbal medicine, on the survival of mice infected with influenza virus

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Abstract

The antiviral effect of Hochu-ekki-to (TJ-41), a Japanese herbal medicine, was investigated using mice infected with influenza virus. TJ-41 was found to increase the survival rate, prolong the mean survival days, suppress viral growth in bronchoalveolar labage fluid (BALF) and inhibit the lung index (lung consolidation) on day 4 after infection in mice infected with influenza, after the agent had been administered orally once daily from day 7 to 2 before infection and from day 0 to 4 after infection. Administration of TJ-41 decreased the BALF concentrations of IL-1α, IL-6 and GM-CSF, but not TNF-α or interferon-γ (IFN-γ), on day 4 after infection. In addition, TJ-41 elevated the level of IFN-α in BALF on day 2 after infection. Yet, TJ-41 did not show any inhibitory effect on the growth of influenza virus in vitro. These results suggest that TJ-41 exerts its inhibitory effect on influenza virus infection *via* enhancement of the host immune responses in this experimental murine system.

Keywords: Hochu-ekki-to; TJ-41; Influenza virus; Inflammatory cytokines; Interferon

1. Introduction

Influenza viruses frequently cause major epidemics of respiratory diseases. Traditional herbal medicines have been used to treat influenza infection for 2000 years in China. Recently, several kinds of these herbal medicines have been used clinically for various diseases in Japan. Hochu-ekki-to (TJ-41) is one such herbal medicine used for patients with bacterial infections, viral infections or postsurgical shock. On the other hand, several workers have reported that TJ-41 promotes certain biological activities such as enhancement of NK activity (Cho et al., 1991), mitogenic activity (Iwama et al., 1986; Kaga et al., 1991), macrophage activity (Kataoka et al., 1989), and recovery of bone marrow cells in irradiated

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mice (Hosokawa, 1986). Although TJ-41 is able to enhance the host immune responses, few basic studies regarding its antiviral effect have been reported. In this paper, we examined the antiviral effect of TJ-41 in mice infected with influenza virus and we also investigated the kinetics of appearance of cytokines such as IL-1α, IL-6, GM-CSF, TNF-α and interferon (IFN) in bronchoalveolar lavage fluid (BALF).

2. Materials and methods

2.1. Chemicals

TJ-41, consisting of spray-dried hot water extracts of a mixture of ten medicinal plants, was obtained from Ibaraki Plant of our company Tsumura & Co., Tokyo, Japan. TJ-41 consists of *Astragali radix* (16.7%), *Atractyloclis lanceae rhizoma* (16.7%), *Ginseng radix* (16.7%), *Angelicase radix* (12.5%), *Bupleuri radix* (8.3%), *Zizyphi fructus* (8.3%), *Aurantii nobilis pericarpium* (8.3%), *Glycyrrhizae radix* (6.3%), *Cimicifugae rhizoma* (4.2%) and *Zingiberis rhizoma* (2.0%). Mouse IFN was obtained from Japan Chemical Research Co., Ltd. Kobe, Japan. Anti-mouse IFN-α/βS and anti-mouse IFN-α were purchased from Pasel + Lorei GmbH and Co., Frankfurt, Germany and Yamasa Shoyu Co., Ltd., Chiba, Japan, respectively.

2.2. Analysis of TJ-41 by high performance liquid chromatography (HPLC)

TJ-41 (0.5 g) was extracted with 20 mL of methanol under ultrasonication for 30 min followed by centrifugation. Analysis of the supernatant solution was performed by high performance liquid chromatography (HPLC) using two LC-10AD pumps, an SPD-M10AVP photodiode-array detector, and a CTO-10AC column oven Shimazu, (Tokyo, Japan) with a TSK GEL ODS-80TS column 250×4.6 mm (Tosoh, Tokyo, Japan) The solvents were (A) 0.05 M ammonium acetate–acetic acid buffer (pH 3.6); (B) 100% acetonitrile. A linear gradient of 90% A and 10% B changing over 1 h to 0% A and 100% B was applied (0% A and 100% B was continued for 20 min). The flow rate and the column temperature were 1.0 ml/min at 40°C, respectively. The UV data of the effluent from the column ranging from 200 to 420 nm was collected and the peak analysis and assignment were performed using system analysis software, CLASS-LC10 (Shimadzu).

2.3. Animals and viruses

Male BALB/cByj Jcl mice were obtained from CLEA Japan Inc., Tokyo, Japan, and acclimatized for at least 1 week before the beginning of the experiments. Mice were 8 weeks old when used. Influenza virus A2 Kumamoto strain (H2N2) was generously supplied by Dr Fujio Suzuki, University of Texas, Galveston, TX, USA, Vesicular stomatitis virus (VSV, New Jersey strain) was obtained from the National Institute of Animal Health, Ministry of Agriculture, Forestry and Fisheries, Tokyo, Japan. The viruses were stored at −70°C until used.

2.4. Influenza virus infection and preparation of BALF

Groups of 10 or 11 mice were used. Mice were infected intranasally at approximately 2 LD_{50}/mouse in a volume of 10 μL under anesthesia with ether. TJ-41 was dissolved in distilled water, and then further diluted with water to the appropriate concentration. TJ-41 solution at the dose indicated was administered orally once daily through a 20-guage feeding needle inserted down the throat of the mice, either before or before and after viral infection was established. Control mice were given distilled water alone. The number of survivors was recorded daily for 21 days after viral infection was established.

BALF was obtained immediately (1 h) before and 2, 4 and 7 days after viral infection. Briefly, mice were killed by exsanguination, then the whole lung was isolated and 1.0 mL of phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) was injected into the trachea with a 21-gauge needle. The solution flowing out was collected and reinjected into the trachea, and this procedure was repeated one more time. The solu-

tion thus obtained was centrifuged at 8000 × *g* for 20 min to remove cells and debris and obtain the supernatant which was designed BALF and stored at −70°C until assayed.

2.5. Virus titer in lung and lung index

Influenza virus titers were determined according to the method reported by Tobita et al. (1975). Briefly, duplicate cultures of MDCK cells in a 6-well plastic plate (Nunc Inc., Roskilde, Denmark) were exposed to 100 μl of BALF samples for 1 h. The cells were overlaid with 2 ml of medium containing 2 μg/ml acetyltrypsin (Sigma Chemical Co., St. Louis, MO), 3% MEM vitamin solution (100x, Gibco BRL Grand Island, New York, NY), 10 mM HEPES (Gibco BRL Grand Island, New York, NY) and 0.6% agar noble (Difco Laboratories, Detroit, MI) followed by cultivation at 35.5°C for 3 days. The cells were overlaid with 2 ml of the above medium containing 0.003% Bacto-neutral red (Difco Laboratories, Detroit, MI) and cultured at 35.5°C for 1 day. The number of plaques was counted. In addition, the lung index was also calculated as a parameter of inflammation or consolidation, according to the following formula:

$$\text{lung index} = \text{lung weight (g)}/\text{body weight (g)} \times 100$$

2.6. Assays for cytokines

IL-1α, IL-6, GM-CSF, TNF-α and IFN-γ in BALF levels were determined by the sandwich enzyme immunoassay using ELISA kits (Amersham International Plc, Buckinghamshire, UK).

2.7. Assay for IFN

IFN titer in BALF was determined by measuring the capacity to inhibit the cytopathic effect of VSV on L-929 cells as described previously (Rubinstein et al., 1981). Mouse IFN, equivalent in titer to the NIH standard reference IFN was used as the standard in each experiment and the IFN titer was expressed in international units per ml (IU/ml).

2.8. Statistical analysis

All of following data were analyzed statistically: survival by χ^2 test and survival days, virus titers, lung index, amount of cytokines and IFN activity by Fisher's protected least significant difference test. If the P value that was obtained was below 0.05, the results were considered significant.

3. Results

3.1. Three-dimensional HPLC profile of TJ-41

The three-dimensional HPLC profile of the methanol solution was shown in Fig. 1. The analysis based on UV-absorption clearly showed the presence of the following major constituents in TJ-41: formonetin (originating from *A. radix*); atractylodinol, acetylatractylodinol and atractylodin (from *A. lanceae rhizoma*); xanthotoxin and ligustilide (from *A. radix*); saikosaponins b1 and b2 (from *B. radix*); narirutin and hesperidin (from *A. nobilis pericarpium*); liquiritin apioside, liquiritin, isoliquiritin apioside, isoliquiritin, liquiritigenin, isoliquiritigenin, formononetin and glycycoumarin (from *Glycyrrhizae*); ferulic acid, cimifugin and caffeic acid (from *C. rhizoma*); 6-gingerol (from *Z. rhizoma*).

3.2. Effect of TJ-41 on influenza virus-infected mice

The effect of TJ-41 on the survival of mice infected with influenza virus was investigated (Fig. 2). In the experiment shown in Fig. 2 (prophylactic/therapeutic treatment), the survival rate of control mice administered distilled water was 40% on day 21 after infection, and TJ-41 at 10, 100 and 1000 mg/kg/day increased the survival rate on day 21 to 80%, 80% and 84%, respectively. While the mean survival days (MSD) of control mice were 13.8 ± 2.1 days, the mice administered TJ-41 at 10, 100 and 1000 mg/kg/day survived for 18.6 ± 1.6 days ($P < 0.05$), 18.9 ± 1.4 days ($P < 0.05$), and 19.0 ± 1.3 days ($P < 0.05$), respectively. When TJ-41 was administered orally once daily for 4 days, starting 1 h after infection (therapeutic

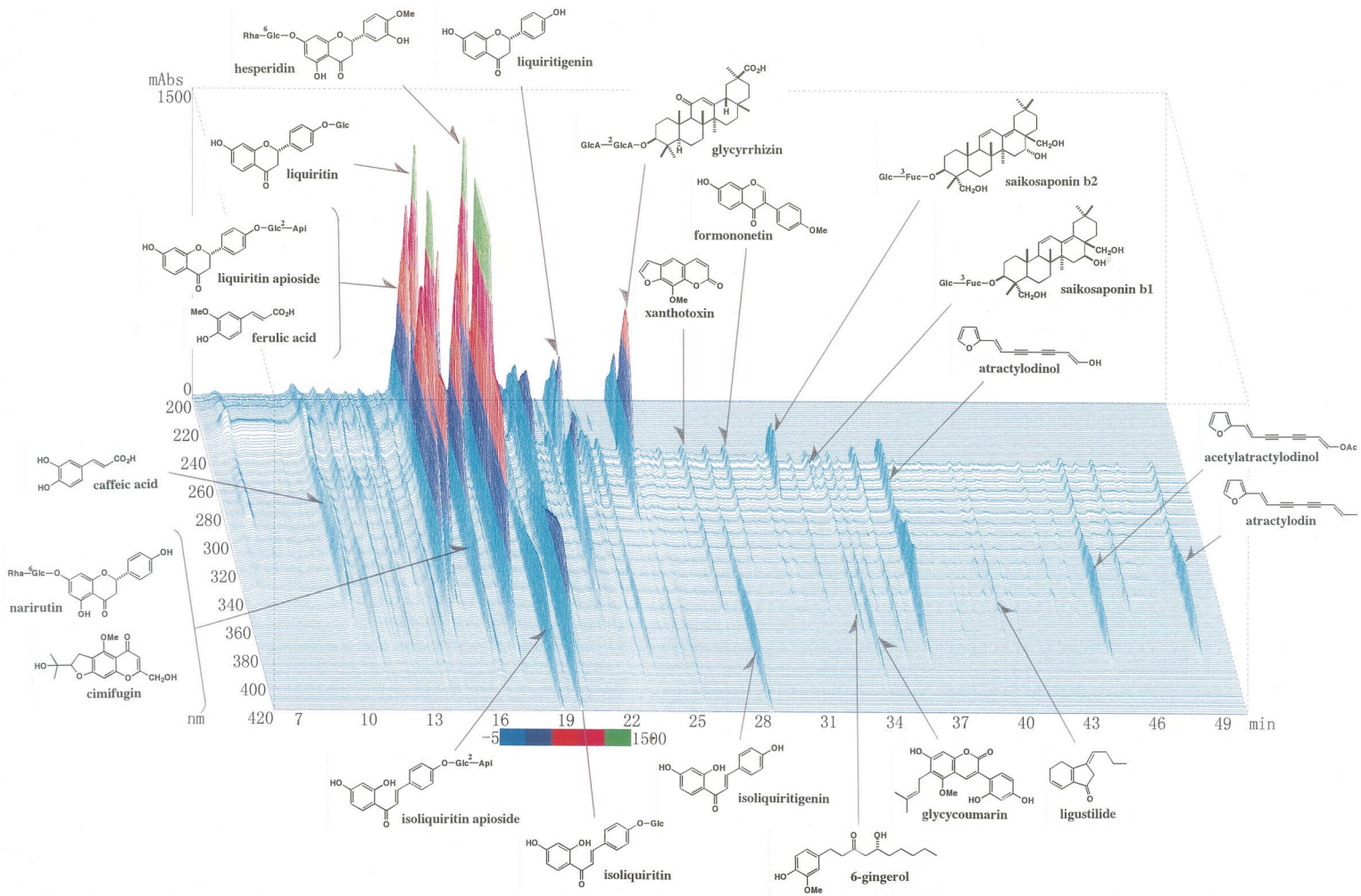

Fig. 1. Analysis of TJ-41 by high performance liquid chromatography (HPLC).

treatment), it showed little effect against influenza virus. The MSD of control, prophylactic/therapeutic treatment of TJ-41 and therapeutic treatment of TJ-41 were 10.5 ± 1.8, 16.8 ± 1.8 days ($P < 0.05$), and 12.6 ± 2.0 days, respectively. These results suggest that using TJ-41 for prophylactic/therapeutic treatment is more effective than its use for therapeutic treatment alone.

The effects of TJ-41 on the growth of the influenza virus in the lung and lung index were investigated (Fig. 3). In mice treated with distilled water alone, virus titers in the lung rapidly increased and peaked 4 days after injection, whereas the lung index continuously increased for as long as 7 days after infection. The virus titers and the lung index of mice treated with TJ-41 were significantly reduced on day 4 compared with those of control mice.

3.3. Kinetics of cytokines in BALF

It is known that some inflammatory cytokines are produced in response to influenza virus infection (Vacheron et al., 1990; Hennet et al., 1992). Therefore, we attempted to measure inflammatory cytokines such as IL-1α, IL-6, GM-CSF, TNF-α and IFN-γ in BALF of influenza virus-infected mice administered TJ-41 or distilled water. As shown in Table 1 IL-1α, IL-6, GM-CSF, TNF-α were produced in BALF from control mice during influenza virus infection. The levels of cytokines produced peaked at 2 or 4 days after infection, except for IL-6 and IFN-γ which remained elevated until 7 days after infection. The level of cytokines in the BALF samples ,except for TNF-α and IFN-γ, from mice administered TJ-41, was significantly reduced on day 4 compared with those in control mice.

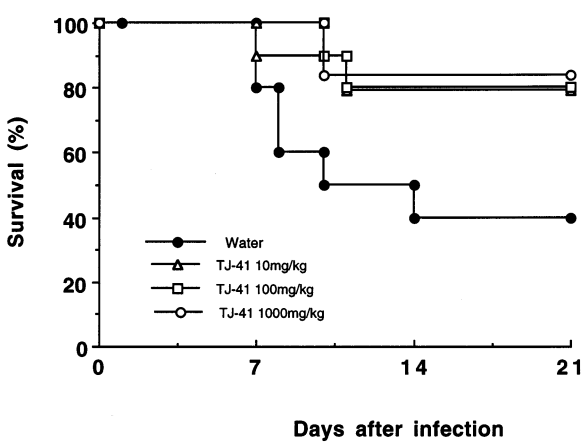

Fig. 2. Effect of TJ-41 on the survival of influenza virus-infected mice. The administration schedules of TJ-41 were as follows: TJ-41 at doses of 10, 100, and 1000 mg/kg was administered orally once daily from day 7 to 2 before infection and continued from day 0 to 4 after infection.

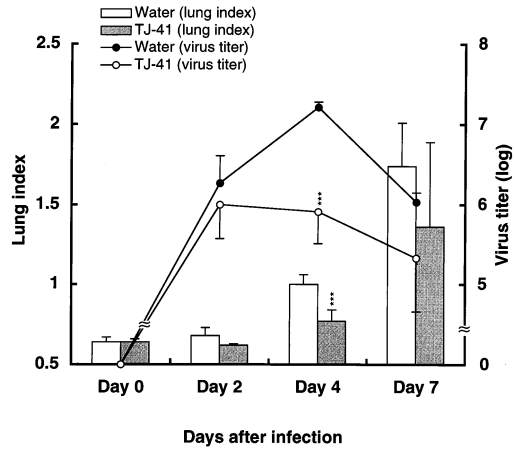

Fig. 3. Influenza virus titer in the lung and consolidations of influenza virus-infected mice administered 1000 mg/kg of TJ-41 or distilled water. TJ-41 was administered orally once daily from day 7 to 2 before infection and continued from day 0 to 4 after infection. Samples were obtained from mock-infected mice (day 0) and from virus-infected mice on day 2, 4 or 7 after infection. Values represent the means of data obtained from four mice. *** $P < 0.001$ compared with control mice on each day after infection.

3.4. Induction of IFN

To examine the possible induction of IFN by TJ-41, BALF samples were taken for the IFN assay. In influenza virus-infected mice treated with distilled water alone, the IFN level was slightly increased after 2 days but markedly increased after 4 days, and then decreased by 7 days after infection (Fig. 4). In mice treated with TJ-41, the IFN level was significantly enhanced 2 days after infection compared with that in the control group and a peak was also seen at 4 days after infection, but the peak level was much lower than

Table 1
Kinetics of the appearance of cytokines in BALF

Days after infection[a]	Sample	IL-1α (pg/ml)	IL-6 (pg/ml)	GM-CSF (pg/ml)	TNF-α (pg/ml)	IFN-γ (pg/ml)
Day 0	Water	1.2 ± 0.4[b]	14.9 ± 9.5	26.1 ± 3.3	164.8 ± 18.8	0
	TJ-41 1000 mg/kg	0.7 ± 0.2	33.8 ± 14.7	27.1 ± 0.7	147.6 ± 16.1	0
Day 2	Water	5.0 ± 4.4	807.5 ± 543.4	41.3 ± 22.7	379.7 ± 48.8	178.2 ± 44.2
	TJ-41 1000 mg/kg	6.5 ± 3.4	825.4 ± 350.1	43.4 ± 7.7	376.1 ± 41.3	207.0 ± 136.0
Day 4	Water	21.1 ± 3.1	2168.9 ± 385.3	123.4 ± 14.3	305.6 ± 54.6	1818.9 ± 327.0
	TJ-41 1000 mg/kg	6.0 ± 2.5***	388.3 ± 290.2***	54.0 ± 12.8***	224.9 ± 32.9	1448.0 ± 755.2
Day 7	Water	13.1 ± 2.7	5574.5 ± 191.3	30.0 ± 2.4	105.0 ± 29.5	3206.8 ± 67.0
	TJ-41 1000 mg/kg	6.6 ± 1.9	3494.8 ± 1518.4	23.8 ± 4.7	173.2 ± 47.5	2443.5 ± 747.4

[a] TJ-41 was administered orally once daily from day 7 to 2 before infection and continued form day 0 to 4 after infection. BALF samples were obtained from mock-infected mice (day 0) and from influenza virus-infected mice at day 2, 4 or 7 after infection.
[b] Values represent the means of data for four mice. *** $P<0.001$ compared with control mice. P values were determined by Fisher's test.

that in the control group. Similar results were obtained in another experiment (data not shown). To determine the nature of IFN obtained from TJ-41-administered mice on day 2, we examined BALF samples using an antibody-neutralisation test antibody. The samples were treated with anti-mouse IFN-α/β antibody or anti-mouse IFN-α antibody at 4°C for 1 h. This IFN activity (320 ± 113 IU/ml) was neutralized by both anti-mouse IFN-α/β antibody and anti-mouse IFN-α antibody to < 10 IU/ml. Therefore, we conclude that the IFN induced by TJ-41 on day 2 is predominantly IFN-α.

Next, we investigated the effect of TJ-41 on the survival of influenza virus-infected mice treated with anti-mouse IFN-α/β (Table 2). The mice administered distilled water, and treated with anti-mouse IFN-α/β (group 1) tended to die earlier than mice not treated with anti-mouse IFN-α/β (group 2), but the survival rate on day 21 after infection was the same. The mice administered TJ-41 (group 3) demonstrated prolonged survival times and 90% of the mice were protected against influenza, while mice treated with anti-mouse IFN-α/β (group 4) reduced the survival rate, IFN level and virus titer as compared with group 3. This result strongly suggests that IFN augmentation by TJ-41 on day 2 after infection is important in its antiviral activity.

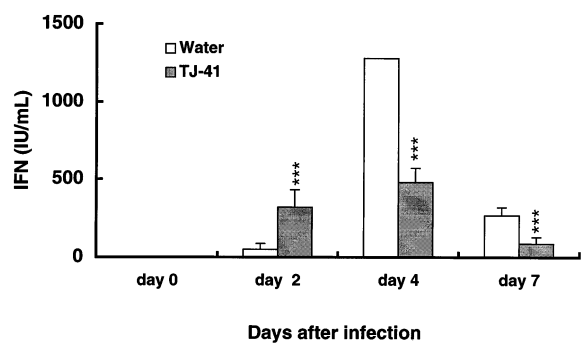

Fig. 4. IFN in BALF of influenza virus-infected mice administered with 1000 mg/kg of TJ-41 or distilled water. TJ-41 was administered with 1000 mg/kg of TJ-41 or distilled water. TJ-41 was administered orally once daily form day 7 to 2 before infection and continued from day 0 to 4 after infection. Samples were obtained from mock-infected mice (day 0) and from infected mice on day 2, 4 or 7 after infection. *** $P<0.001$ compared with control mice on each day after infection.

Table 2
Effect of TJ-41 on survival of influenza virus-infected mice treated with anti-IFN

Group[a]		Day 2 after infection[c]		MSD[d]	S/T[e]
	Anti-IFN α/β[b]	IFN	Virus titer		
1. Water	(−)	120 ± 80	6.8 ± 0.4	15.5 ± 1.9	5/10
2. Water	(+)	60 ± 40	6.6 ± 0.5	14.7 ± 2.1	5/10
3.TJ-41 1000 mg/kg/day	(−)	760 ± 610	6.0 ± 0.1	19.9 ± 1.1*	9/10*
4.TJ-41 1000 mg/kg/day	(+)	40 ± 20	7.1 ± 0.5	13.4 ± 2.1*	4/10*

[a] TJ-41 or water was administered orally once daily from day 7 to 2 before infection and continued from day 0 to 4 after infection.
[b] Anti-IFN α/β (about 1000 IU/mouse/day) was injected intravenously into mice on days −1, 0 and +1 relative to viral infection.
[c] IFN (IU/ml) and virus titer (PFU/ml) in BALF were obtained 2 days after viral infection.
[d] MSD, mean survival days.
[e] S/T, surviving mice over total animals tested on 21st day after infection. * $P<0.05$, all values represent the means ± S.E.

4. Discussion

It is known that the pathogenicity of the influenza virus in mice is caused by over-reaction of the immune response of the host rather than the direct effect of viral replication (Oda et al., 1989). The immune response induces infiltration of lymphocytes, neutrophils and macrophages in lung tissue (Hers et al., 1962). These calls produce certain kinds of inflammatory cytokines, which then cause inflammation in the lung, and this inflammation can induce lethal damage. Inflammatory cytokines such as IL-1 (Oppenheim et al., 1986), IL-6 (Nishimoto et al., 1989), GM-CSF (Gasson, 1991) and TNF-α (Sherry and Cerami, 1988) may play an important role in influenza virus-induced pathogenesis. We have now measured these cytokines in BALF of influenza virus-infected mice administered TJ-41 or distilled water. As shown in Table 1, levels of IL-1α, IL-6, GM-CSF and TNF-α of mice administered distilled water were increased after infection. Similar observations were recently reported by Hennet et al. (1992) and Vacheron et al. (1990). However, levels of IL-1α, IL-6 and GM-CSF in infected mice that had received TJ-41 were significantly lower than those in control mice on day 4 after infection. These reductions may have contributed to the increased survival rate of influenza virus-infected mice treated with TJ-41. It is of interest that neither TNF-α or IFN-γ production was affected by TJ-41, even if the reason remains unclear.

It is well known that IFN is particularly important in limiting the spread of influenza virus infection (Isaacs and Hitchock, 1960; Stewart, 1979; Garcia-Sastre et al., 1998). It has also been reported that the peak level of IFN in BALF was detected on day 4 after influenza virus infection (Wyde et al., 1982). Our data also demonstrated that IFN in BALF was detectable from day 2 through 7 after infection with a peak on day 4 (Fig. 4). The patterns of IFN production were similar in mice treated with either TJ-41 or distilled water. However, the IFN level in mice treated with TJ-41 was significantly higher than that in control mice on day 2 (Fig. 4). TJ-41 may have an adjuvant activity on IFN production since TJ-41 does not have IFN-inducing activity by itself. The antiviral activity of TJ-41 disappeared when the anti-mouse IFN-α/β antibody neutralized IFN augmentation by TJ-41 on day 2 after infection (Table 2). Therefore, we conclude that the augmentation of IFN in the early stage of infection plays an important role in the antiviral activity of TJ-41. Similar results were found with regard to other biological activities of TJ-41 by Shimizu et al. (1997).

The effect of TJ-41 on the production of IFN-γ in cultured splenocytes was tested using tumor-bearing mice inoculated with *Salmonella enteritidis*. Spleen cells prepared from mice treated with TJ-41 produced high levels of IFN compared with the treatment of distilled water, but TJ-41 itself did not stimulate IFN-γ induction. In addition,

Ikeda et al. (1985) reported that a synthetic muramyldipeptide and its analogues showed augmentation of early-type serum IFN production in mice induced by either LPS or polyI:polyC, although the muramyldipeptide (analogues) did not have any IFN-inducing activity by themselves (Ikeda et al., 1985).

We also attempted to measure NK activity in the lung, but the number of cells harvested was not sufficient. Therefore, we measured NK cell activity using spleen cells. As a result, NK activity in control mice apearred from day 2 through 7 after infection with a peak on day 4. NK activity in mice administered TJ-41 did not significantly differ from that in the control (data not shown). Alterations of T-cells (Thy 1.2 positive cells) and B-cells (B220 positive cells) in spleen cells, were also analyzed by FACScan (Becton Dickonson Co., Ltd.). The population of these cells obtained from mice administered TJ-41 did not change compared with that in the control (data not shown).

TJ-41 at concentrations of 2–1000 μg/ml did not inhibit the growth of influenza virus in vitro (data not shown). It thus appears that TJ-41 has antiviral activity in vivo, but not in vitro. Recently, we found that TJ-41 may be effective in immunocompromised mice infected with herpes simplex virus type I. Our data indicate that TJ-41 helped stimulating IFN in the mouse after viral infection. However, the precise mechanism by which TJ-41 stimulates the host's own antiviral functions remains obscure. Further studies are required to elucidate this issue.

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