ANTIVIRAL AND INTERFERON-INDUCING ACTIVITIES OF A NEW PEPTIDOMANNAN, KS-2, EXTRACTED FROM CULTURE MYCELIA OF *LENTINUS EDODES*

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Oral (PO) administration of KS-2 to adult DDI mice resulted in a peak serum interferon (IF) titer of 800 units (U)/ml 20 hours after administration with detectable levels persisting until 30 hours. After intraperitoneal (IP) injection, a peak serum IF titer of 1,600 U/ml was detected and it followed the same time course as that of oral administration. The IF induced by KS-2 shared certain physico-chemical properties with the standard preparation of immune IF and was not neutralized by an antiserum against type I IF. In mice infected intranasally (IN) with influenza A_2 (H_2N_2) virus, KS-2 was found to possess significant protective activities. Efficacy of the agent was evidenced by an increase in survivor number, a prolongation of mean survival time, an inhibition of the development of lung consolidation induced by the viral infection and a decrease in virus titer in lung tissues. Both PO and IP administrations of KS-2 protected mice against infection and significant antiviral activities were achieved not only by prophylactic but also chemotherapeutic administration. No virocidal or virostatic activities of KS-2 to the influenza virus were found *in vitro*. The protective activities of KS-2 against influenza virus infection in mice are discussed in view of the immunopotentiation of the host animals.

Chemotherapy of viral infections including influenza and herpes viruses is a formidable task. Antiviral agents without toxicity but exhibiting highly therapeutic value have been intensively searched in many laboratories for a long time. We have carried out *in vivo* screening for a protective substance against influenza diseases using a mouse model which acts through a potentiation of the defense mechanisms of the host animal rather than a direct inhibition of the viral growth. Three kinds of IF inducer, *viz.*, a natural double-stranded RNA (S-dsRNA) extracted from the spores of *Lentinus edodes*[1,2], dextran phosphate (DP-40) prepared by a chemical phosphorylation of dextran[3] and a glutarimide antibiotic (9-methylstreptimidone, 9-MS) isolated from a culture filtrate of *Streptomyces* sp. S-885[4,5], have been discovered during the screening program. These inducers are unique in their characteristics. For example, S-dsRNA accomplishes a high level of protection against influenza A/SW-15 virus infection in mice[1,2], whereas a potent IF inducer of a synthetic double-stranded RNA, polyriboinosinic-polyribocytidylic acid, is almost ineffective against such influenza A infection[6]. Both DP-40[3] and 9-MS[5] induce higher titers of IF in lung tissue, the site of influenza virus multiplication than in the serum. The protective effects and the stimulation of IF production, however, are observed, only when these inducers are given IP or intravenously (IV) to mice[1~5].

Recently, we observed IF production to be induced by both PO and IP administrations of a new antitumor agent, KS-2, which was extracted from culture mycelia of *Lentinus edodes* as an immunopotentiator. KS-2 possesses α-mannose linked to a small peptide[7] with an estimated molecular weight of $6\times10^4\sim9.5\times10^4$. When given either PO or IP, KS-2 suppressed the growth of various transplanted tumors in mice and rats through the potentiation of the host's function and not through direct inhibition of growth of the tumor cells. The acute LD_{50} of the immunopotentiator is more than 12,500 mg/kg when administered PO to mice[7]. This fact may indicate the potential application of KS-2 in man. The purpose of this paper is to describe the IF-inducing capacity of this immunopotentiator and its protective activity against influenza virus infection in mice.

Materials and Methods

Drug preparation

KS-2 used in this study was extracted from culture mycelia of *Lentinus edodes*. Its characteristics and isolation procedure were reported previously[7]. The final product, a white amorphous lyophilized powder, was dissolved in sterile 0.01 M phosphate-buffered 0.15 M saline (PBS, pH 7.4) immediately before use and 0.5 ml of the solution containing an appropriate concentration was injected into mice. Virazole[8] (1-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) was used as the positive control for an anti-influenza drug. Purified protein derivative (PPD), which was used as a stimulator of type II IF induction, was kindly supplied by Dr. SUGANUMA, Mitsui Pharmaceutical Co., Ltd., Mobara, Chiba, Japan.

Mice

White DDI mice of both sexes weighing 18~20 g were used throughout the study. They were obtained from the Central Farm of Tohoku University where care was taken to prevent infections with Sendai virus and *Mycoplasma*.

Cells

Monolayer cultures of the thymidine-kinaseless mutant strain of mouse fibroblasts, L-1D cells, were grown in ROUX bottles in EAGLE's minimal essential medium (MEM) supplemented with 10% bovine serum and used as the target cells for IF titration. Primary cultures of human embryonic lung (HEL) cells grown in MEM supplemented 10% calf serum were used to examine the host species specificity of the IF induced by KS-2.

Viruses

For the IF assay, the Indiana strain of vesicular stomatitis virus (VSV), propagated in the L-1D cells and stored at −80°C, was used as a challenge virus. The 50% tissue culture infectious dose ($TCID_{50}$) of VSV was $10^{7.0}$/ml in these cells. The mouse adapted Kumamoto strain of influenza A_2 (H_2N_2) virus was used for the infection[9]. The virus was propagated once in the allantoic cavity of embryonated chicken eggs and the 48-hour allantoic fluid was used as the inoculum. The 50% egg infectious dose (EID_{50}) of the fluid was $10^{9.2}$/ml, which corresponded to $10^{5.0}$ LD_{50} in mice inoculated IN according to our standard inhalation procedure[10].

IF

The tube culture assay technique (RYTEL and BALAY[11]) with a minor modification was employed for the measurement of serum IF obtained from KS-2 treated mice. Briefly, the L-1D cells were seeded 3 days before the test so that each tube contained $1.8\sim2.0\times10^5$ cells. Serial 2-fold dilutions of serum specimens obtained from KS-2 treated mice were made in maintenance medium (MEM and 2% calf serum) and added to monolayer cultures of L-1D cells. After 18~24 hours of stationary incubation at 37°C, the medium was aspirated and the cultures washed once with HANKS' balanced salt solution. A dose of 100 $TCID_{50}$ of VSV was inoculated into each tube. After 20~24 hours of stationary incubation at 37°C, the cytopathic effect (CPE) in the treated tubes was compared with the controls. The IF titers were determined as the reciprocals of the highest dilution of the specimens that produced 50%

protection of the CPE at a time when the virus controls showed 100% CPE.

Antisera and IF standards

Two kinds of standard mouse IF were used for a determination of the characteristics of serum IF induced by KS-2 in mice. One of them is a type I standard IF[12] which was kindly supplied by Dr. KOBAYASHI, Basic Research Laboratory, Toray Industries Inc., Kamakura, Japan. This IF was prepared *in vitro* using large-scale suspension cultures of mouse L cells and Newcastle disease virus (NDV) system. The other is a type II (or immune) IF reported by YOUNGNER and SALVIN[13]. Briefly, mice were inoculated into tail vein with 0.1 ml of a bacterial suspension containing 5×10^6 to 10^7 viable *Mycobacterium tuberculosis* strain BCG cells. Two to 3 weeks after infection, mice were administered IV with a 0.5 mg/kg dose of PPD. Three hours after PPD challenge, mice were bled and obtained serum specimens were used as the immune IF standard. Crude rabbit anti-serum, prepared against type I IF induced in mouse L cells by NDV, was a gift from Dr. KAWADA, Institute for Virus Research, Kyoto University, Kyoto, Japan. For neutralization testing, 1 ml of serum IF (800 U/ml) obtained from mice 20 hours after KS-2 injection was incubated at 37°C for 1 hour with 1 ml of anti-serum to the interferon at a final dilution of 1: 10. The mixture was then added to L-1D cells for IF assay.

Mouse virus infection

Virus infection was made by inhalation of the influenza virus. A vaponephrine-type nebulizer was connected to the compressor to spray about 10 ml of diluted allantoic fluid in 30 minutes. This procedure resulted in the inoculation of 0.002 ml of the fluid per mouse[10] containing 10 or 20 LD_{50} of virus. The 50% lethal dose (LD_{50}) was determined according to the REED and MUENCH procedures.

Determination of the amount of virus in lung tissue by EID_{50} and hemagglutinin (HA) titration

The lungs of infected mice were disrupted by a polytron homogenizer (Swiss Kinematica Co., Ltd., Switzerland) and made up to a 10% suspension with nutrient broth. The suspension was then clarified by centrifugation at 1,580 *g* for 10 minutes. The supernatants were serially diluted 10-fold with broth and 0.2 ml of each dilution was inoculated into the allantoic cavities of 3 embryonated eggs. After incubation for 72 hours at 36°C, the allantoic fluid was removed from each egg and tested for its hemagglutinin titration titer by SALK's pattern method. The EID_{50} per organ was calculated by the method of REED and MUENCH.

Calculation of lung consolidation score

Before autopsy, the lungs of the infected mice were irrigated by intracardiac injection of 0.01 M phosphate buffer (pH 7.2) to remove trace amounts of red blood cells. The amount of consolidation was scored macroscopically according to the method of HORSFALL[14].

Evaluation of antiviral activity in mice

The drug dissolved in PBS was administered PO or IP on a milligram per kilogram basis according to the schedule. The criteria for anti-influenza activity were based on: (1) An increase in the mean survival time of mice succumbing to infection during the 30-day observation period; (2) an increase in the number of survivors on day 30; (3) a decrease in the virus titer in lung tissues; (4) an inhibition of the lung consolidation induced by the viral infection. The results obtained were evaluated statistically by the means of the STUDENT's *t* (average of survival days) and chi-square (survival %) tests. If the *P* value obtained in each test was below 0.05, the drug dose was considered to have had a significant antiviral activity.

In vitro antiviral test

In the CPE inhibition test, L-1D cells were grown in test tubes, and KS-2 of appropriate concentrations was added to the cultures at the time of VSV infection in a dose of 100 TCD_{50}. Uninfected control tubes were always included to determine toxicity of each concentration of KS-2. After incubation at 37°C, these cultures were examined daily for virus induced CPE. Antiviral activity of the compound was determined when control cultures (infected, without KS-2) showed complete CPE. In the virus yield-reduction test, embryonated eggs were infected with influenza A_2/Kumamoto (H_2N_2) virus at a dose of 10 EID_{50}, and then 0.2 ml per egg of broth with or without KS-2 was injected to the allantoic cavity. Forty eight hours after incubation at 37°C, these eggs were harvested at 4°C for 18 hours. And obtained

allantoic fluids were assayed for their virus titers by EID_{50} method.

Results

IF-inducing Activity of KS-2 in Mice

In order to detect the IF-inducing capability of KS-2, serum specimens were obtained at various time intervals after IP or PO administration of a 200 mg/kg dose of KS-2 and assayed for antiviral activity. As shown in Fig. 1, the antiviral activity appeared by 16 hours and reached a peak of 1,600 U/ml by IP injection and 800 U/ml by PO administration 20 hours after treatment. In the next experiment, the dose response effect was examined with serum specimens obtained 20 hours after IP injection with increasing doses, ranging from 0.2 to 200 mg/kg, of KS-2 (Table 1). The antiviral activities were induced in the sera of mice injected with more than the 2 mg/kg dose. In the range from 2 to 200 mg/kg, however, the production of the antiviral activity was not dependent on the dose administered.

Fig. 1. Time course of the induction of serum IF by KS-2 in mice.

At various times after IP or PO administration with a 200 mg/kg dose of KS-2, five mice from each group were bled by decapitation and the sera were assayed for antiviral activity.

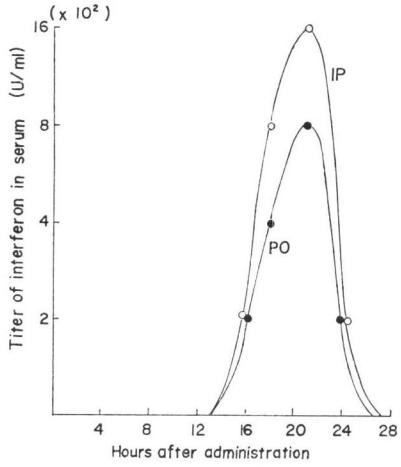


Table 1. Dose response of KS-2 on induction of serum IF in mice.

Dose (mg/kg)	Titer of IF* (U/ml)
200	1,600
20	1,600
2	800
0.2	20
0	20

* Sera obtained 20 hours after IP injection with various doses of KS-2 were assayed for their antiviral activities.

Characterization of Serum IF Induced by KS-2 in Mice

To determine the biological and physicochemical properties of IF induced by KS-2, pooled sera were obtained from mice 20 hours after injection. Dilutions of sera were exposed to monolayer cultures of HEL cells for comparison with L-lD cells. The development of CPE caused by VSV challenge was inhibited significantly in L-1D cells but not in HEL cells. In contrast, the serum specimens obtained from a GRAWITZ tumor-patient administered 20 hours after KS-2 (200 mg/kg, IP) inhibited the viral CPE in HEL cells. Thus, KS-2 induced viral in-

Table 2. Species specificity of IF activity induced by KS-2.

Specimen obtained from:	Titer of IF (U/ml)[a]	
	HEL cells	L-1D cells
Human[b]	1,250	<20
Mouse[c]	<20	1,600

a) Titer was measured by the reciprocal of the serum dilution which gave a 50% protection against a challenge of 100 $TCID_{50}$ of VSV.

b) The human specimen was obtained from a patient with GRAWITZ's tumor 20 hours after PO administration with a 200 mg/kg dose of KS-2.

c) Pooled mouse-serum IF was obtained from 10 mice 20 hours after IP injection with a 200 mg/kg dose of KS-2.

hibitor is host-species specific (Table 2). The antiviral activity was readily degraded by trypsin treatment. The activity was also inactivated significantly by both acid dialysis and heat treatment (Table 3). KS-2 induced IF could not be neutralized by an anti-IF serum specific for type I IF and fully active against NDV-induced IF (Table 4).

Protective Effect of KS-2 against Influenza Infection in Mice

Two groups of 25 mice, one group of 20 mice and one group of 50 mice were simultaneously infected IN with 10 LD_{50} of influenza A virus. The first two groups received KS-2 at a dose of 200 mg/kg by IP and PO administrations, respectively, three times, 24, 3 and 1 hour before and twice, 1 and 3 hours, after viral infection and then twice daily for 4 consecutive days. The third group was given 50 mg/kg dose of virazole and the fourth group was given 0.5 ml PBS which served as positive and negative controls, respectively. As shown in Table 5, whereas 100% of the mice receiving PBS died within 17 days (mean survival days: 9.2), 52% of the mice treated with IP doses of KS-2 survived for the observation period of 30 days (mean survival days: >20.0). Oral administration of KS-2 and IP administration of virazole gave survival rates of 32% (mean survival days: >19.0) and 30% (mean survival days: >16.9), respectively. The increases in survival rate of these mice were statistically significant when compared with the negative control. The mean survival time of mice treated with KS-

Table 3. Some characteristics of three kinds of mouse IF.

Treatment	Titer of IF (U/ml) (% loss of activity)		
	Serum IF induced by KS-2[a]	Type I IF[b]	Type II IF[c]
Trypsin[d], 500 mcg/ml, 37°C, 2 hours	<20 (>98.8)	<20 (>95)	<20 (>96.7)
PBS, 37°C, 2 hours	1,600	400	600
Temperature[e], 56°C, 1 hour	<20 (>98.8)	400 (0)	80 (86.7)
Temperature, 37°C, 1 hour	1,600	400	600
pH 2.0[f], 4°C, 20 hours	<20 (>98.8)	400 (0)	<20 (>96.7)
pH 7.2, 4°C, 20 hours	1,600	400	600

a) IF samples were prepared from the sera of mice which were injected IP with a 200 mg/kg dose of KS-2.
b) Type I IF was produced *in vitro* by a L cells and NDV system.
c) Serum specimen obtained from BCG-sensitized mice 3 hours after IV injection of PPD at a dose of 0.5 mg/kg was used as a standard type II IF.
d) IF samples containing a 500 mcg/ml concentration of trypsin were incubated at 37°C for 2 hours.
e) Resistance to heat was determined by exposing sera to 56°C for 1 hour at neutral pH.
f) Dialysis against pH 2.0 buffer was conducted at 4°C for 20 hours and was followed by redialysis against PBS (pH 7.2) for 20 hours.

Table 4. Failure of neutralization of KS-2-induced serum IF by anti-type I IF rabbit serum.

Treatment[a]	IF	Titer of IF (U/ml)[b] (% loss of activity)
Anti-type I IF serum	Type I IF[c]	<20 (>95)
Normal rabbit serum	Type I IF	400 (0)
Anti-type I IF serum	Type II IF[d]	600 (0)
Normal rabbit serum	Type II IF	600 (0)
Anti-type I IF serum	Serum IF induced by KS-2[e]	400 (0)
Normal rabbit serum	Serum IF induced by KS-2	400 (0)

a) One ml of anti-type I IF serum (1: 10 diluted) or the same amount of normal rabbit serum was added to 1 ml IF samples and incubated at 37°C for 1 hour.
b) After incubation at 37°C for 1 hour, these mixtures were assayed for residual IF activities in L-1D cells.
c) Mouse type I IF was produced *in vitro* by a L cells and NDV system.
d) Mouse type II IF was prepared by BCG-sensitized mice and PPD system.
e) Serum specimens were obtained 20 hours after KS-2 administration.

Table 5. Effect of KS-2 on influenza virus infection[a] in mice.

Drug[b]	Dose (mg/kg)	Route of administration	No. of mice	Mean survival days[c] (*P*)[d]	Survival %[e] (*P*)[f]
KS-2	200	IP[g]	25	>20.0 (<0.001)	52 (<0.01)
KS-2	200	PO[h]	25	>19.0 (<0.001)	32 (<0.01)
Virazole	50	IP	20	>16.9 (<0.001)	30 (<0.01)
PBS	(0.5 ml)	PO	50	9.2	0

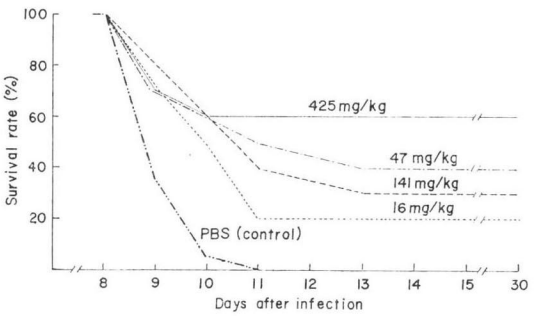
a) Ten LD_{50} of influenza virus was challenged by inhalation.
b) The drugs were given according to the schedule: 24, 3 and 1 hour before, 1 and 3 hours after infection and twice daily for 4 consecutive days.
c) Mean survival days were calculated by day 30 after infection.
d) Probability value obtained by use of STUDENT's *t* test.
e) Percentage of survivors on day 30 postinfection.
f) Probability value obtained by use of chi-square analysis.
g) Intraperitoneal
h) Per os

2 was also significantly increased.

Different doses were used to obtain more details of the protective effect of KS-2. Four groups of 10 mice and one group of 39 mice were infected with 20 LD_{50} of influenza virus and the mice of the first four groups were given PO 16 to 425 mg/kg doses of KS-2, according to the schedule described above.

As shown in Fig. 2, the group treated with a 425 mg/kg dose of KS-2 had a survival rate of 60%, whereas all of the control mice treated with PBS died within 11 days of virus infection. Protection was demonstrated at all the doses but it seems that the protection does not depended the administered doses. The mean survival time of the treated mice was also significantly increased.

Fig. 2. Protective effect of PO administration of KS-2 on influenza A_2 (H_2N_2) virus infection in mice.
Mice were challenged IN with 20 LD_{50} of the virus and given PO the indicated doses of KS-2 three times, 24, 3 and 1 hour, before and twice, 1 and 3 hours after infection and twice daily for 4 consecutive days.



Prophylactic and Therapeutic Effects of KS-2 on Influenza Virus Infection in Mice

The effect of time of administration on the antiviral action of KS-2 was investigated. Three groups of 10 mice and one group of 40 mice were

Table 6. Prophylactic and therapeutic effects of KS-2[a] on influenza virus infection[b] in mice.

Schedule of IP administration	No. of mice	Mean survival days (*P*)	Survival % (*P*)
A. Prophylactic: 2 and 1 day preinfection	10	>20.3(<0.001)	50 (<0.01)
B. Therapeutic: 1, 2, 3, 4 and 5 days postinfection	10	>22.0 (<0.001)	60(<0.01)
2, 3, 4, 5 and 6 days postinfection	10	>16.2 (<0.001)	30 (<0.01)
Not treated	40	9.6	0

a) A 141 mg/kg dose of KS-2 was injected according to schedules.
b) Twenty LD_{50} of influenza virus was challenged IN.
Other experimental conditions and symbols are identical to those in Table 5.

challenged with 20 LD_{50} of influenza virus. The first group of 10 mice was given two IP doses of 141 mg of KS-2/kg prophylactically, 48 and 24 hours before virus infection. Another two groups of 10 mice were also treated chemotherapeutically with the same amount of IP administered KS-2 at different time intervals. Namely, the treatments were initiated 24 or 48 hours after the viral challenge, respectively, and continued once daily for 5 successive days. The last group of 40 mice was given PBS and served as the control. These results are shown in Table 6, in which all of the control mice died within 11 days of virus infection (mean survival days: 9.6), and the group treated in the prophylactic manner gave a 50% survival rate (mean survival days: >20.3). The group treated chemotherapeutically also survived more than 30 days postinfection at the rate of 60% (the group given treatment 24 hours after infection: mean survival days: >22.0) and 30% (the group given treatments 48 hours after infection; mean survival days: >16.2), respectively. Both increases in survival rate and mean survival days of KS-2 treated mice were statistically significant. Therefore the protective effect of KS-2 was demonstrated by chemotherapeutic as well as prophylactic administration.

Effect of KS-2 on the Development of Lung Consolidations in Influenza Infected Mice

Sixty mice were infected IN with 10 LD_{50} of influenza virus. One half of these mice was given a single administration of 200 mg/kg of KS-2 IP, and the other half served as the control. KS-2 was administered 24 hours after virus infection and once daily for 4 consecutive days. Ten mice from both groups were sacrificed at 2-day intervals from day 3 to day 7 for the calculation of lung consolidation scores. As shown in Table 7, although lung lesions were found in all the control mice on day 5 (mean consolidation score: 1.7), almost no lesions were found in the treated group on the same day (mean score: 0.5). On day 7 the lungs of the control group showed almost complete consolidation (mean score: 3.9). In contrast the consolidation score of the treated group on day 7 (mean score: 1.8) was comparable to that of the control on day 5. The difference between the control and treated group was significant (P 0.005) when the 5- and 7-day results were compared. The result of the consolidation score showed a delay of about 2 days in the treated group.

Table 7. Effect of KS-2 on the development of lung consolidation in influenza virus[a] infected mice.

KS-2[b] (mg/kg)	Consolidation score[c]		
	3 days[d]	5 days	7 days
200	trace	0.5	1.8
0	0.5	1.7	3.9

a) Ten LD_{50} doses of influenza virus were challenged by inhalation.
b) KS-2 was given IP 24 hours after virus infection and once daily for 4 consecutive days.
c) The grade of consolidation was determined according to the method of HORSFALL.
d) Days after virus infection.

Table 8. Effect of KS-2 on the growth of influenza virus in mouse lung.

KS-2 (mg/kg)	Virus titer /organ (log_{10} EID_{50})[a]		
	3 days	5 days	7 days
200	4.7	6.2	5.2
0	7.5	8.1	5.0

a) A titration procedure of influenza virus is described in Materials and Methods section in the text.

Other experimental conditions are identical to those in Table 7.

Virus Titrations in Lung Tissues of Infected Mice

Growth of influenza virus in lung tissues was examined in the infected mice treated with and without KS-2. The administration schedule of KS-2 was the same as that in the consolidation experiment and all the mice were infected IN with 10 LD_{50} of the virus. Sixty mice were divided into two

groups. One group was treated IP with KS-2 at the dose of 200 mg/kg and the other received 0.5 ml of PBS per mouse IP. Ten mice from both groups were sacrificed at 2-day intervals, from day 3 to 7, for virus titration. The results, as shown in Table 8, indicate a delay in the initial growth of influenza virus which is equivalent to almost 10^2 reduction of the maximum titer in the treated group. Thus the virus growth in the lung tissues in control mice reached $10^{8.1}$ EID_{50}/organ 5 days after infection. In contrast, the virus titer in the lung tissues of KS-2 treated mice was $10^{6.2}$ EID_{50}/organ on the same day.

Possible Direct Action of KS-2 on Virus Particles or Virus Infected Cells

To test the possibility of direct interactions of KS-2 with the virus particles, 1 ml of 10^6 $TCID_{50}$ of VSV per ml or the same amount of 10^7 EID_{50} of influenza virus was mixed with 1 ml of broth containing 2~4 mg of KS-2 per ml, respectively, and incubated for 5 hours at 37°C. Control virus preparations containing formalin instead of KS-2 were incubated similarly. The initial and residual virus contents were determined by titrating the VSV preparation obtained from L-1D cells and the influenza samples in the chorioallantoic cavity of chick embryos. As shown in Table 9, KS-2 did not reveal any virocidal effect on VSV and influenza virus. On the CPE inhibition test in L-1D cells infected with VSV and the virus-yield reduction test in embryonated eggs infected with influenza A_2/Kumamoto (H_2N_2) virus, KS-2 did not show any inhibitory action on the growth of these viruses when examined at a concentration of 500 mcg/ml. Thus KS-2 proved to possess neither virocidal nor virostatic activity *in vitro*.

Table 9. Lack of virocidal activity of KS-2 on influenza A_2 (H_2N_2) virus and VSV.

Material	Concentration	Residual activity of:	
		Influenza virus (EID_{50}/ml)	VSV ($TCID_{50}$/ml)
KS-2	2 mg/ml	2.5×10^6	2.5×10^5
KS-2	1 mg/ml	2.5×10^6	2.5×10^5
Formalin	50%	$<10^2$	$<10^2$
Broth	—	2.5×10^6	2.5×10^5

Note: One ml of 10^6 $TCID_{50}$ of VSV/ml or the same amount of 10^7 EID_{50} of influenza A_2 (H_2N_2) virus was mixed with 1 ml of broth containing 2~4 mg/ml of KS-2, formalin and broth alone, respectively. Five hours after incubation at 37°C, each mixture was titrated for the residual activity of the viruses.

Discussion

Several natural and synthetic immunopotentiators, including IF inducers such as polyriboinosinic-polyribocytidylic acid[6] and pyran[15], and many immunotherapeutic agents for cancer prepared from bacterial cells such as *Mycobacterium tuberculosis* strain BCG[16] and *Corynebacterium parvum*[17], inhibit viral infections *in vivo*. Although the mechanisms of the antiviral action of these immunopotentiators have not been rigorously defined, it is usually understood that the protective effects of IF inducers appear to be due to the IF induced[18,19]. However, the protective action of the immunotherapeutic agents remains to be clarified although the activated reticuloendothelial and cellular immunity system might be involved[20,21].

Recently KIRCHNER *et al.*[22,23] reported that killed *Corynebacterium parvum* induced an immune IF *in vivo* as well as *in vitro*. More recently we observed the IF-inducing activity by some immunotherapeutic agents[24~26] such as a whole cell preparation of an avirulent *Streptococcus pyrogenes* (OK-432)[27], an antihelminthic drug of thiazole derivation (levamisole)[28] and a high molecular weight β-1,3 glucan (lentinan) extracted from the fruitbody of *Lentinus edodes*[29].

In the present paper, we examined KS-2 for its IF-inducing capability in experimental animals and for its protective activity against influenza in mice. The results showed that an immune IF was produced by IP or PO administration of KS-2 in mice (Tables 1~4, Fig. 1) and KS-2 exhibited a significant activity against the infections of lethal doses of influenza A_2 (H_2N_2) virus in mice when given either

PO or IP (Table 5, Fig. 2). Significant protection (30%~60% of survival rates) was achieved by therapeutic as well as prophylactic treatment (50% survival rate), whereas 100% mortality was observed in the control group (Table 6). In addition, the marked antiviral activities of KS-2 were seen by decreased titers of the virus in lung tissues (Table 8) and reduced scores of lung consolidations (Table 7) in infected mice. The results showed that KS-2 itself had no direct action on virus particles and virus infected cells *in vitro* (Table 9). Thus KS-2 possesses significant antiviral activity against influenza disease in mice through a potentiation of the host's defense functions and the antiviral action of KS-2 is exhibited through its IF-inducing activity. However, further investigation along this line is required for a more detailed elucidation of the mechanism of action.

In contrast to the viral inhibitory activity, several immunopotentiators enhance a host's tumor suppressive capacity[8,27~30], and the antitumor mechanism has been demonstrated to be engendered by tumoricidal activated macrophages[30,31]. Furthermore SCHULTZ *et al.*[32] reported that macrophages became tumoricidal when incubated *in vitro* with highly purified IF. Our preliminary data indicated that macrophages obtained from mice treated with OK-432, lentinan, levamisole or KS-2 exhibited tumoricidal activity. Taking all these observations together, the antiviral and antitumor activities of the immunopotentiators may be explained through the function of IF. More detailed analysis on the relationships between the IF-inducing capability and the antitumor or antiviral activity of the immunopotentiators should be carried out.

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