# PROTECTIVE EFFECTS OF IMMUNOACTIVE PEPTIDE, FK565 AGAINST SYSTEMIC AND LOCAL INFECTIONS WITH HERPES SIMPLEX VIRUS AND MURINE CYTOMEGALOVIRUS AND RESPIRATORY TRACT INFECTION WITH INFLUENZA VIRUS IN MICE

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The protective effects of FK565 against systemic infections with herpes simplex virus (HSV) and murine cytomegalovirus (MCMV), respiratory tract infection with influenza virus and zosteriform rash with HSV investigated in mice. FK565 showed excellent protective activities against systemic infections with both acyclovir (ACV)-sensitive and -resistant HSV at intravenous and subcutaneous doses of 0.1 and 1 mg/kg and oral dose of 1 mg/kg. FK565 showed superior protective activities at subcutaneous doses of 0.01 and 0.1 mg/kg compared to ACV at subcutaneous dose of 15 mg/kg against MCMV infection. In respiratory tract infection with influenza virus, FK565 showed potent protective effects at intravenous, subcutaneous and oral doses of 0.001 to 1 mg/kg. FK565 markedly inhibited zosteriform spread of HSV on the flank skin at an intravenous dose of 0.1 mg/kg and the mice given FK565 survived longer than the control mice. The peritoneal exudate cells from FK565-treated mice suppressed the growth of HSV in mouse embryo fibroblast more strongly than those from the control mice, although FK565 had no direct antiviral activity against HSV. These findings suggest that FK565 enhanced the host defense ability against viral infections by nonspecific activiation of macrophages.

Infections with herpes simplex virus (HSV) are ubiquitious in man and are usually locally restricted. However, they may cause generalized infections in immuno-incompetent newborns and in immunodeficient or immunosuppressed patients[1]. The most common diseases are recurrent episodes of orallabial or genital disease that are localized on cutaneous or mucosal surfaces. HSV is a common cause of fatal and sporadic encephalitis. HSV is also suspected to be an oncogenic agent, particularly in the development of cervical carcinoma[2~5].

Human cytomegalovirus (HCMV), one of the most cmmon viruses causing intrauterine infections in man, causes infections in about 0.4%~2.3% of all live births[6]. Transplacental infection of the fetuses results from either a primary maternal infection or the reactivation of a maternal infection during pregnancy. Most congenital infections rsulting from a reactivated maternal infection are asymptomatic[7]. However, after a primary maternal infection during pregnancy, 40%~50% of the fetuses are infected and 10%~15% of these fetuses develope an apparent disease[8,9].

Several new nucleoside analogs have been developed which are potent inhibitors of herpes simplex virus (HSV) with low toxicity *in vivo*[10,11]. 9-(2-Hydroxyethoxymethyl)guanine (ACV) and *E*-5-(bromovinyl-2′-deoxyuridine) (BVDU) behave as analogs of thymidine and are selectively phosphorylated by the HSV-specified pyrimidine deoxynucleoside kinase (thymidine kinase, TK). Both drugs are then metabolized within the infected cells to their respective nucleoside triphosphates which then interfere with HSV-specified DNA polymerase and inhibit virus replication. Resistance to ACV has been associated

with HSV mutants which have completely lost the ability to induce the viral TK. In an immunocompromised patients or patients with complex disease, a satisfactory therapy for HSV infection dose not exist, and attempts to specifically immunize against HSV have not been encouraging in terms of effectiveness. Therefore, it appears useful to screen nonspecific stimulants of the immune system for their antiviral activity.

We reported in previous papers that FK565, an immunoactive peptide was isolated from culture filtrates of *Streptomyces olivaceogriseus*[12~14] and the compound induced potent resistance against microbial, fungal and viral infections in normal and immunosuppressed mice[15~18], *in vivo* tumoricidal activity[19,20], inhibitory effect against murine tumor metastases[21], and a broad spectrum of immunostimulatory activities such as adjuvant effects on humoral and cellular immunity and mitogenesis[22].

In this study, we investigated the protective effect of FK565 using animal infection models with HSV, murine cytomegalovirus (MCMV) and influenza virus.

## Materials and Methods

### Drug

FK565 was sunthesized at the Product Development Laboratories, Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan. Acyclovir (Lot. No. SC136) was supplied from Wellcome-Foundation Ltd., London.

### Animal

Male ICR strain mice aged 4 weeks in groups of 20 or 3 weeks in groups of 10 were used; male Balb/c nu/nu strain mice aged 6 weeks in groups of 7 were used. The animals were obtained from Japan SLC, Inc. and housed in an isolation room at 21~25°C upon reciept at our institution and supplied with food and water *ad libitum*.

### Virus

Herpes simplex virus (HSV) type 1 (strains Miyama and Fukuda, acyclovir-sensitive) and type 2 (strain CL706, acyclovir resistant), murine cytomegalovirus (MCMV, salivary gland homogenates from mice infected with MCMV) and influenza virus (strain A/PR8) were used in these experiments. These viruses were stored at −70°C until used.

### Experimental Infection

Systemic Infection: HSV strains Miyama and CL705 were used to induce encephalitis in ICR strain mice aged 4 weeks in groups of 20. Mice were challenged intraperitoneally with $2.8 \times 10^3$ pfu/mouse (0.2 ml/mouse) of strain Miyama and with $1.6 \times 10^4$ pfu/mouse (0.2 ml/mouse) of strain CL705. The surival rate of the infected animals was observed for 14 days after challenge. ICR strain mice aged 3 weeks in groups of 10 were challenged intraperitoneally with 0.2 ml/mouse ($2.9 \times 10^5$ pfu/mouse) of MCMV. The survival rate of animals was observed for 10 days after challenge.

Respiratory Tract Infection: Influenza virus strain A/PR8 was used to induce respiratory tract infection. 25 μl of viral suspension was intranasally inoculated to ICR strain mice aged 4 weeks in groups of 20, under pentobarbital anesthesia. The survival rate of animals was observed for 14 days after challenge and the lung consolidations were observed on days 4, 7 and 10 after challenge. The lung consolidations were graded according to the severity as follows: Grade 0; negative, 1; 10~20%, 2; 20~40%, 3; 40~60%, 4; 60~80% and 5; 80~100% or death.

Skin Infection: HSV strain Fukuda was used to induce zosteriform rash on the flank in athymic nude mice, Balb/c nu/nu. Mice were intradermally challenged at an abdominal site with $3.7 \times 10^7$ pfu/mouse (50 μl/mouse). Zosteriform spread of skin lesion was observed after challenge of virus for 10 days. This model is particularly useful for estimation of viral spread along neurons because the zosteriform rash is an epiphenomenon that accompanies infection along the peripheral nervous system. The skin

lesions were graded according to the severity as follows: Grade 0; negative, 1~3; vesicle, 4~6; moderate (zosteriform spread), 7~9; severe (zosteriform rash) and 10; death.

Dosing Schedule

In systemic infection with HSV or respiratory tract infection with influenza virus, FK565 at doses of 0.001 to 1 mg/kg was given intravenously, subcutaneously or orally 4 hours after challenge and thereafter once a day for 4 consecutive days (total 5 times). In systemic infection with MCMV, FK565 at doses of 0.001 to 1 mg/kg was given subcutaneously one day before challenge and one and 3 days after challenge (total 3 times). ACV at doses of 5, 10 and 15 mg/kg was given subcutaneously twice a day for 4 consecutive days after challenge (total 8 times). These doses of ACV were those used clinically. In skin lesions with HSV, FK565 was given intravenously 4 hours after challenge and thereafter once a day for 4 consecutive days (total 5 times).

Plaque Assay

*In Vitro* Antiviral Activity: Monolayers of embryonic fibroblast from ICR mice were cultured in EAGLE's minimum essential medium (MEM) containing 10% fetal bovine serum (FBS) in a 24 well-multiwell tray at 37°C in 5% $CO_2$. 20 pfu of HSV strain Miyama were adsorbed to the cells for 1 hour at 37°C in 5% $CO_2$. The cells were washed with 5% FBS-MEM and thereafter cultured in 5% FBS-MEM containing 1.0 to 100 $\mu$g/ml of FK565 and 0.5% human immunoglobulin for 3 days at 37°C in 5% $CO_2$. The infected cells fixed with 5% trichloroacetic acid were stained with 0.1% crystal violet and the plaques were counted by microscopy. The mean values were obtained from 4 preparations.

*Ex Vivo* Antiviral Activity: FK565 of 0.001 to 1 mg/kg was given intravenously once a day for 3 consecutive days in groups of 8 ICR strain mice and the peritoneal exudate cells (PEC) were collected from the peritoneal cavity and were pooled in each dose on the next day of final dosing. Monolayers of embryonic fibroblasts ($2 \times 10^5$ cells/well) from ICR mice were infected with HSV strain Miyama at 0.1 pfu/cell and thereafter co-cultured with those PEC ($2 \times 10^6$ cells/well) from FK565-treated mice for 20 hours at 37°C in 5% $CO_2$. The infected cells were scraped, sonicated and the viral yields were titrated by the plaque method using Vero cells. The mean values of HSV titers were obtained from 3 preparations.

## Results

### Protective Effect of FK565 against Systemic Infection with HSV in Mice

The results from systemic infections with ACV-sensitive and -resistant strains, Miyama and CL705 are shown in Table 1. In the intravenous and subcutaneous dosing groups for strain Miyama, 55% to 70% of the infected animals given 0.1 and 1 mg/kg of FK565 survived, and the survival rates were su-

Table 1. Protective effect of FK565 against ACV-sensitive- and -resistant-herpes simplex virus infections in mice.

| HSV, strain (inoculation: cfu/mouse) | FK565 route | Survival rate (%) | | | | |
|---|---|---|---|---|---|---|
| | | 0 | 0.001 | 0.01 | 0.1 | 1 mg/kg |
| Miyama ($2.8 \times 10^3$) | IV | 0 | 0 | 25 | 65* | 70* |
| | SC | 5 | 0 | 15 | 60* | 55* |
| | PO | 5 | 0 | 0 | 15 | 30** |
| CL705 ($1.6 \times 10^4$) | IV | 0 | 0 | 15 | 60* | 80* |
| | SC | 0 | 15 | 15 | 75* | 75* |
| | PO | 0 | 0 | 5 | 0 | 15 |

HSV (strain Miyama or CL705) was intraperitoneally inoculated to ICR mice aged 4 weeks in groups of 20.
FK565 or ACV was given intravenously, subcutaneously or orally 4 hours after challenge and thereafter once a day for 4 consecutive days (total 5 times).
Survival rates were observed for 14 days after challenge.
Significant difference from the control (* $P<0.05$, ** $P<0.1$) by Chi square test.

perior to that of the control with significant differences ($P<0.05$). In the oral dosing groups, the survival rate at 1 mg/kg was 30% and superior to that of the control. In the intravenous and subcutaneous dosing groups for strain CL705, 60% to 80% of the infected animals given 0.1 and 1 mg/kg survived, and the survival rates were superior to those of the control. In the oral dosing groups, the survival rate at 1 mg/kg was low and FK565 had no effect.

### Protective Effect of FK565 against Systemic Infection with MCMV

The results are shown in Table 2. The survival rates at doses of 0.01 and 0.1 mg/kg were superior to that of the control. However, FK565 had no effect at 0.001 mg/kg and lower survival rate at 1 mg/kg than that at 0.1 mg/kg or 0.01 mg/kg suggested toxicity of the drug. The potent protection was observed when FK565 was given both before and after MCMV challenge in mice, but not when FK565 was given only after challenge. When ACV was given at 10 and 15 mg/kg twice a day after challenge, the survival rates were superior to that of the control.

Table 2. Protective effect of FK565 against murine cytomegalovirus infection in mice.

| Drug | Dose (mg/kg/time) | Survival rate (%) |
|---|---|---|
| FK565 | 0 | 0 |
| | 0.001 | 20 |
| | 0.01 | 80* |
| | 0.1 | 70* |
| | 1 | 40* |
| ACV | 5 | 0 |
| | 10 | 50* |
| | 15 | 50* |

Murine cytomegalovirus (MCMV) was intraperitoneally inoculated to ICR mice aged 3 weeks in groups of 10.

FK565 was given 0.001 to 1 mg/kg subcutaneously once a day 1 day before challenge and 1 and 3 days after challenge (total 3 times).

ACV was given 5 to 15 mg/kg subcutaneously twice a day 1, 2, 3 and 4 days after challenge (total 8 times).

Survival rates were observed for 10 days after challenge.

Significant difference from the control (* $P<0.05$) by Chi square test.

Fig. 1. Inhibitory effect of FK565 against lung consolidation with respiratory tract infection by influenza virus in mice.

● Control, ■ 0.01 mg/kg, ▲ 0.1 mg/kg, △ 1 mg/kg. Significant difference from the control by $t$-test (* $P<0.05$).

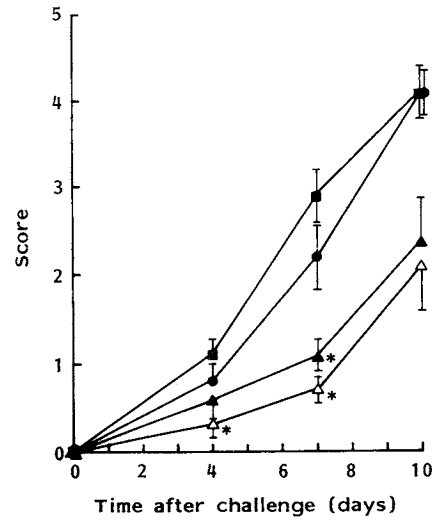


Table 3. Protective effect of FK565 against influenza virus infection in mice.

| Drug | Route | Survival rate (%) | | | | |
|---|---|---|---|---|---|---|
| | | 0 | 0.001 | 0.01 | 0.1 | 1 mg/kg |
| FK565 | IV | 10 | 45* | 45* | 50* | 50* |
| | SC | 20 | 20 | 50** | 45 | 45 |
| | PO | 15 | 30 | 35 | 40 | 50** |

Influenza virus was intranasally inoculated to ICR mice aged 4 weeks in groups of 20.

FK565 was given 0.001 to 1 mg/kg intraperitoneally, subcutaneously and orally 4 hours after challenge and once a day for 4 days (total 5).

Survival rates were observed for 14 days after challenge.

Significant difference from the control (* $P<0.05$, ** $P<0.1$) by Chi square test.

### Protective Effect of FK565 against Respiratory Tract Infection with Influenza Virus

The results are shown in Table 3 and Fig. 1. The animals were observed for 14 days after challenge. In the intravenous dose groups, the survival rates were 45 to 50% at doses of 0.001 to 1 mg/kg and were superior to that of the control. The survival rates were 45 to 50% in the subcutaneous dosing groups of 0.01 to 1 mg/kg and 40 to 50% in the oral dosing groups of 0.1 to 1 mg/kg. The protective effect by intravenous dosing was the most potent among subcutaneous and oral dosing.

The inhibitory effects of FK565 on lung consolidations by influenza virus were dose-dependent when FK565 was given intravenously.

### Inhibitory Effect of FK565 against Zosteriform Rash by HSV on Flank Skin

The results are shown in Fig. 2. Zosteriform spread of skin lesion with HSV in mice was inhibited markedly at 0.1 mg/kg of FK565 but not at 0.01 mg/kg. 85% of the untreated control mice died within 10 days after HSV challenge. On day 10 after challenge, 60% of the animals given 0.1 mg/kg survived but all animals given 0.01 mg/kg died within 10 days.

### Lack of Direct Antiviral Activity of FK565

FK565 in concentrations of 1.0 to 100 $\mu$g/ml did not decrease HSV plaque formation in a plaque assay method (Table 4). The results indicated that FK565 has no direct artiviral activity against HSV, even at a concentration of 100 $\mu$g/ml.

### Inhibitory Effect of Peritoneal Exudate Cells from FK565-treated Mice

When the viral growth of HSV was examined in the presence of peritoneal exudate cells (PEC)

Fig. 2. Inhibitory effect of FK565 against zosteriform spread on skin by herpes simplex virus in athymic nude mice.

● Control, ■ 0.01 mg/kg, ▲ 0.1 mg/kg.
Significant difference from the control by *t*-test (* $P<0.05$).

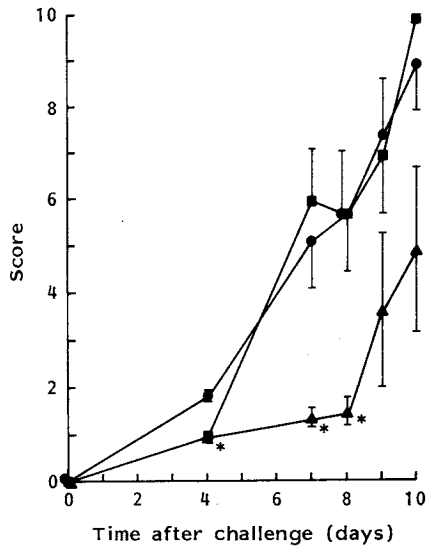


Table 4. Lack of direct antiviral activity of FK565 against herpes simplex virus in mouse embryo fibroblast.

| Concentration ($\mu$g/ml) | No. of plaque (n=4) |
|---|---|
| 0 | 17.5±0.7 |
| 1.0 | 16.0±1.1 |
| 3.2 | 16.8±1.0 |
| 10 | 15.3±1.3 |
| 32 | 16.5±1.3 |
| 100 | 18.5±2.1 |

The mean values were obtained from 4 preparations.

Table 5. *Ex vivo* antiviral activity of peritoneal exudate cells of mice given FK565 against herpes simplex virus.

| Dose of FK565 (mg/kg) | No. of PEC treated with FK565 ($\times 10^6$ cells/mouse) | Mean HSV titer (pfu/well) | Inhibition (%) |
|---|---|---|---|
| 0 (Saline) | 2.3 | $5.0\times10^5$ | 0 |
| 0.0001 | 4.2 | $3.7\times10^5$ | 26 |
| 0.001 | 7.2 | $2.1\times10^{5}$* | 58 |
| 0.01 | 18.2 | $1.9\times10^{5}$* | 62 |
| 0.1 | 34.6 | $1.4\times10^{5}$* | 72 |
| 1 | 32.4 | $1.8\times10^{5}$* | 64 |

The mean values were obtained from 3 preparations.
Significant different from control (saline-treated mice) by *t*-test (* $P<0.05$).

previously activated by FK565 *in vivo*, FK565 showed dose-dependent inhibition of viral yield of HSV (Table 5). 58% to 72% inhibitory effects were observed at a dose of 0.001 to 1 mg/kg with significant difference from the control ($P<0.05$). The optimum dose was 0.1 mg/kg (72% inhibition).

## Discussion

Our experiments demonstrated the striking protective effect of FK565 in lethal encephalitis induced by two strains of HSV, one is ACV-sensitivity ($ID_{50}$: strain Miyama; 0.38 $\mu$M) and one highly resistant to ACV ($ID_{50}$: strain CL705; 36 $\mu$M). The protective effect of immunoactive peptide, FK565 against both ACV-sensitive and -resistant HSV infection was dose-dependent. The effective doses of FK565 were 0.1 to 1 mg/kg by the intravenous and subcutaneous routes and 1 mg/kg by oral route against the two strains. The potent inhibition of HSV infection by FK565 was demonstrated by all routes: intravenous, subcutaneous and oral. In the mouse systemic infection with MCMV, FK565 at doses of 0.01 and 0.1 mg/kg had potent protective action. This effect of FK565 was superior to that of ACV at doses of 10 and 15 mg/kg. These results suggest that FK565 may be useful in the treatment of primary maternal infection with HCMV during pregnancy. Against respiratory tract infection with influenza virus, the survival rates by FK565 ranged from 35% to 45% at doses of 0.01 to 1 mg/kg intravenously, subcutaneously or orally. The lung consolidations were mild in mice treated with FK565 at 0.1 or 1 mg/kg when compared with that in control mice.

FK565 showed inhibition on viral growth of HSV in the presence of peritoneal exudate cell previously activated by FK565 *in vivo* but could not inhibit that of HSV directly *in vitro*. These results suggest that FK565 possibly has no direct virucidal activity, but rather enhances the host defense ability, particularly macrophages against viral infection.

We reported that FK565 afforded resistance in normal or immunosuppressed mice against bacterial, fungal and friend leukemia virus infections by activating natural killer cells[23], macrophages and the reticuloendothelial system[24,25], and also by inducing colony stimulating factors[26] or interleukin 1 production[27,28]. Moreover some of these activities maintained for 5 to 7 days after a single dose. Therefore, the inhibitory activity of FK565 against viral infection appears to be due to activation of host defense mechanisms. FK565 may have clinical potential if the mouse data can be applied to humans although toxicities in man are unknown.

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