IMMUNOACTIVE PEPTIDES, FK-156 AND FK-565. II

RESTORATION OF HOST RESISTANCE TO MICROBIAL INFECTION IN IMMUNOSUPPRESSED MICE

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The immunoactive peptides, FK-156 and its analogue, FK-565 were evaluated in various models of mice immunosuppressed with cyclophosphamide, hydrocortisone, mitomycin C, carrageenan and tumor cells. Treatment with FK-156 (subcutaneous) and FK-565 (oral) markedly restored host defense ability against microbial infection. The therapeutic effect of ticarcillin or gentamicin alone against pseudomonal infection in cyclophosphamide- and hydrocortisone-treated mice and tumor-bearing mice was much lower than in normal mice. The therapeutic effect of these antibiotics against pseudomonal infection in immunosuppressed mice was enhanced markedly by combined use with FK-156. The killing ability of macrophages and polymorphonuclear leukocytes of the immunosuppressed mice was also markedly enhanced by dosing with FK-156.

In recent years, the incidence of serious infection in immunocompromised hosts has increased, despite the use of antibiotics with potent antibacterial activity in these patients. These phenomena are mainly due to decreased host resistance to microbial invasion. We have reported in the preceding paper that the immunoactive peptide, FK-156 and its analogue FK-565 enhanced host defense ability against microbial infection in normal mice.[1] The present study focuses on decrease of host resistance against microbial infections in various types of immunosuppressed mouse models, the restorative effect of FK-156 and FK-565 in impaired host defense and the synergistic effect of FK-156 and various antibiotics.

Materials and Methods

Drugs

FK-156 (D-lactoyl-L-alanyl-γ-D-glutamyl-(L)-*meso*-diaminopimelyl-(L)-glycine) and FK-565 (heptanoyl-γ-D-glutamyl-(L)-*meso*-diaminopimelyl-(D)-alanine) were synthesized in the Fujisawa Research Laboratories. FK-156 and FK-565 were given respectively to mice in subcutaneous and oral single or multiple doses of 1 mg/kg and 0.1 mg/kg before microbial challenge.

Immunosuppressants

Cyclophosphamide (Endoxan, Shionogi & Co., Ltd.), hydrocortisone (Nakarai Chemical Ltd.), carrageenan (Sigma Chemical Company), mitomycin C (Kyowa Hakko Kogyo Co., Ltd.) and sarcoma 180 in ascites form was used. Cell-free supernatant of sonicated sarcoma 180 ascites was prepared as described by PIKE and SNYDERMAN[2].

Antibiotics

Ticarcillin (Beecham Research Laboratories) and gentamicin (Schering Corp.) were used.

Immunosuppressed Mice

Male ICR strain mice aged 4 weeks, unless otherwise specified, were used in groups of 10. Cyclophosphamide and mitomycin C were given in a single intraperitoneal dose of 200 mg/kg and 3 mg/kg, respectively 4 days before challenge. Carrageenan was given in a single intraperitoneal dose of 60 mg/kg 2 days before challenge. Hydrocortisone was given in subcutaneous doses of 50 mg/kg once a day for 4 days before challenge. Sarcoma 180 tumor cells were implanted intraperitoneally at a concentration of 6.0×10^6 cells per mouse (*dd*Y-strain) 8 days before challenge.

Infection in Mice

Pseudomonas aeruginosa and *Listeria monocytogenes* were inoculated intraperitoneally, and *Candida albicans* and *Salmonella enteritidis* were inoculated intravenously into various types of immunosuppressed mice; in tumor-bearing mice, *P. aeruginosa* was inoculated intravenously. Host resistance to infection was expressed as survival rate.

Synergistic Protection Test of FK-156 and Antibiotics

FK-156 was given twice in subcutaneous doses of 1 mg/kg/dose 3 and 1 day before challenge. *P. aeruginosa* strain 97 was inoculated intraperitoneally into the cyclophosphamide- and hydrocortisone-treated mice, and intravenously into the tumor-bearing mice. The test antibiotics were given in subcutaneous doses 1 and 3 hours after challenge. The protective effect of the antibiotics was expressed as ED_{50} values from the number of surviving mice 6 days after challenge.

Under the same dosing and challenge schedules, the viable cell counts in the liver, blood and peritoneal cavity of immunosuppressed mice after combined therapy with ticarcillin and FK-156 were compared with those after dosing with the drugs alone. The mice were used in groups of 5. The cyclophosphamide-treated mice and tumor-bearing mice were bled to death 16 hours after challenge and the hydrocortisone-treated mice at 10 hours. The peritoneal fluid with the exception of tumor-bearing mice was collected from the peritoneal cavity after intraperitoneal injection with 5 ml of sterile saline. Serial 10-fold dilutions of the blood, peritoneal fluid and liver homogenate were prepared with saline and the samples were plated on Heart Infusion Agar (Difco). The number of colony forming units was determined after 24 hours of incubation at 37°C.

Phagocytosis and Killing

Peritoneal macrophage suspension was prepared by the method described by BJORSON[8]. 0.25 ml of phagocyte suspension, 0.1 ml of ticarcillin solution, 0.05 ml of anti-pseudomonal serum, and 0.1 ml of *P. aeruginosa* suspension were placed in a siliconized glass tube with a rubber stopper. This mixture contained about 8.0×10^6 phagocytes, 5 μg (1/10 the MIC) of ticarcillin, 10% immune serum and about 1.0×10^5 cfu of *P. aeruginosa* per ml in HANKS' balanced salt solution. The mixture was incubated at 37°C for 2 hours with rotation (4 rpm). The total viable cells were plated on agar to count colony forming units.

In the experiment using sonicated tumor cells, peritoneal PMN were obtained from normal mice 3 hours after peritoneal injection of 2.0 ml of 0.5% glycogen solution. 10% supernatant of sonicated tumor cells and 10 μg/ml of FK-156 were added to the above *in vitro* incubation system.

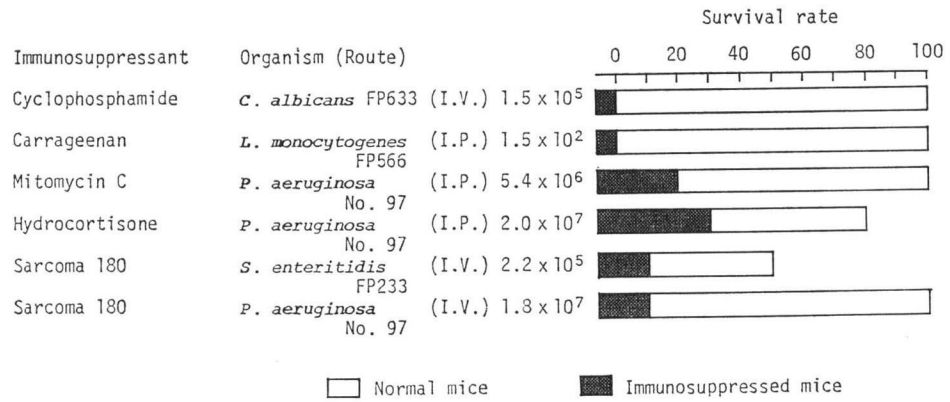
Results

Decreased Host Defense against Microbial Infection in Immunosuppressed Mice

The abilities of normal and immunosuppressed mice to survive when challenged with microbial infections are compared in Fig. 1. In this experiment, the immunosuppressed mice were prepared by dosing with immunosuppressants such as cyclophosphamide, carrageenan, mitomycin C, hydrocortisone or by implantation with sarcoma 180.

All cyclophosphamide-treated mice died after challenge with *C. albicans* strain FP633 (1.5×10^5 cfu/

Fig. 1. Subversion of host resistance to microbial infection in immunosuppressed mice.



mouse); however, under the same conditions, all the normal mice survived. The same phenomenon was seen in the carrageenan-treated and normal mice with *L. monocytogenes* infection. The survival rates of the mitomycin C-treated and hydrocortisone-treated mice against *P. aeruginosa* infection were 20 and 30%, respectively. In contrast, the survival rates of normal mice were 80% or more. The survival rates of tumor-bearing mice with sarcoma 180 against infection due to *S. enteritidis* strain FP233 (i.v.) or *P. aeruginosa* (i.v.) were both 10% as compared with 50% and 100% in normal mice respectively.

Although the immunosuppressants used in this experiment all differ in mechanism of impairing immunological function in animals, without exception, they markedly decreased the ability of mice to handle various microbial infections.

Effects of FK-156 and FK-565 on Host Resistance to Microbial Infection in Immunosuppressed Mice

The restorative effects of FK-156 and FK-565 on host defense abilities in immunosuppressed mice were investigated. FK-156 and FK-565 were given to immunosuppressed mice in subcutaneous and oral doses of 1 and 0.1 mg/kg respectively before challenge. As shown in Table 1, the survival rates of the

Table 1. Effect of FK-156 and FK-565 on defensive ability of immunosuppressed mice against microbial infection.

Immunosuppressant	Organism	Treatment (Day)	Dose (mg/kg)	% Survival	
				FK-156	FK-565
Cyclophosphamide	*C. albicans* FP633	−6, −5, −2, −1	0.1	30	40
			Control	0	0
Carrageenan	*L. monocytogenes* FP566	−6, −5, −2, −1	0.1	80	60
			Control	0	0
Mitomycin C	*P. aeruginosa* No. 97	−3	1	70	100
			Control	30	20
Hydrocortisone	*P. aeruginosa* No. 97	−3, −1	1	40*	70*
			Control	0	0
Sarcoma 180	*S. enteritidis* FP233	−6, −5, −4, −1	1	70	60
			Control	10	10
Sarcoma 180	*P. aeruginosa* No. 97	−3, −1	1	60	100
			Control	10	30

FK-156 and FK-565 were given subcutaneously and orally in doses of 0.1 and 1 mg/kg respectively on the day given above (*intraperitoneally).

mice treated with FK-156 were significantly higher than those of the non-treated mice. Without exception, FK-156 afforded increased host resistance to systemic infections with extracellular and facultative intracellular parasites in immunosuppressed mice. A similar effect was also seen with the oral doses of FK-565.

Synergistic Effect of FK-156 and Antibiotics on Enhancement of Host Resistance to *P. aeruginosa* Infection in Immunosuppressed Mice

The protective effects of ticarcillin and gentamicin alone against pseudomonal infection in 3 types of immunosuppressed mice were compared with those in normal mice. The therapeutic effect of these antibiotics alone was significantly lower in all the immunosuppressed mice than in the normal mice (Table 2). The protective effect of these antibiotics in combination with FK-156 against infection in immunosuppressed mice was investigated under conditions in which FK-156 alone was therapeutically ineffective. The following results were obtained.

Cyclophosphamide-treated Mice

The ED_{50} values of ticarcillin and gentamicin alone against pseudomonal infection in the cyclophosphamide-treated mice were respectively $>1,600$ and 10.4 mg/kg. However, the ED_{50} values of ticarcillin and gentamicin in combination with FK-156 significantly decreased to 771 and 2.81 mg/kg, respectively.

Hydrocortisone-treated Mice

The ED_{50} values of ticarcillin and gentamicin in combination with FK-156 in the hydrocortisone-treated mice significantly decreased from $>1,600$ to 262 mg/kg and from 100 to 9.98 mg/kg, respectively.

Tumor-bearing Mice

The ED_{50} values of ticarcillin and gentamicin alone in the tumor-bearing mice were respectively $>1,600$ and 49.1 mg/kg. However, when the antibiotics were given to infected mice after pretreatment with FK-156, the ED_{50} values significantly decreased to 168 mg/kg for ticarcillin and 7.60 mg/kg for gentamicin.

Table 2. Synergistic protection of antibiotics and FK-156 against pseudomonal infection in immunosuppressed mice.

Immunosuppressant	Antibiotic	ED_{50} (mg/kg)		
		Normal mice	Immunosuppressed mice	
			—	+FK-156
Cyclophosphamide	Ticarcillin	84.6*	>1,600	771*
	Gentamicin	1.37	>1,600 45.0	2.81*
			10.4	
Hydrocortisone	Ticarcillin	107*	813	262*
	Gentamicin	1.19	>1,600 20.4	9.98*
			100	
Tumor-bearing	Ticarcillin	127**	514	168*
	Gentamicin	1.42*	>1,600 3.52	7.60*
			49.1	

FK-156 was given subcutaneously in a dose of 1 mg/kg 3 and 1 day before challenge, antibiotics were given subcutaneously 1 and 3 hours after challenge.

* $P<0.05$, ** $P<0.10$

These results suggest that the therapeutic effect of the antibiotics was enhanced markedly by combined therapy with FK-156 in the immunosuppressed mice tested.

Synergistic Bactericidal Effect of Ticarcillin and FK-156 on Viable Cell Counts in the Peritoneal Cavity, Blood and Liver of Immunosuppressed Mice after Intraperitoneal Challenge with *P. aeruginosa*

Fig. 2 shows that the decrease of viable cells in the peritoneal cavity, blood and liver of infected mice after treatment with ticarcillin and FK-156 was more marked than after treatment with either drug alone.

In the cyclophosphamide-treated mice, the viable cell counts in the peritoneal cavity increased 6.27 log per cavity 16 hours after peritoneal inoculation with *P. aeruginosa* (5.60 log per cavity). Although the viable cell counts after treatment with FK-156 and ticarcillin alone decreased to 4.43 and 4.51 log per cavity, respectively, the decrease after combined therapy was to 2.56 log per cavity. The same tendency was seen in the viable cell counts of the blood and liver. Similar resultants were seen in the hydrocortisone-treated mice and tumor-bearing mice.

These results suggest that the *in vivo* bactericidal effect of the antibiotic was enhanced synergistically by FK-156.

Synergistic Effect of Ticarcillin and FK-156 on Phagocytosis and Killing of *P. aeruginosa* by Peritoneal PMN Leukocytes and Macrophages of Immunosuppressed Mice

Peritoneal macrophages were derived from FK-156-treated and non-treated immunosuppressed mice induced by cyclophosphamide and hydrocortisone. Macrophages and *P. aeruginosa* were incubated at 37°C for 2 hours in the presence or absence of ticarcillin (Fig. 3).

The residual viable cell counts of *P. aeruginosa* were significantly lower when ticarcillin was used in combination with macrophages from immunosuppressed mice treated with FK-156 than when either ticarcillin or the macrophages were used singly.

Fig. 2. Effect of ticarcillin and FK-156 on viable cell counts in peritoneal cavity, blood and liver of immunosuppressed mice challenged with *P. aeruginosa*.

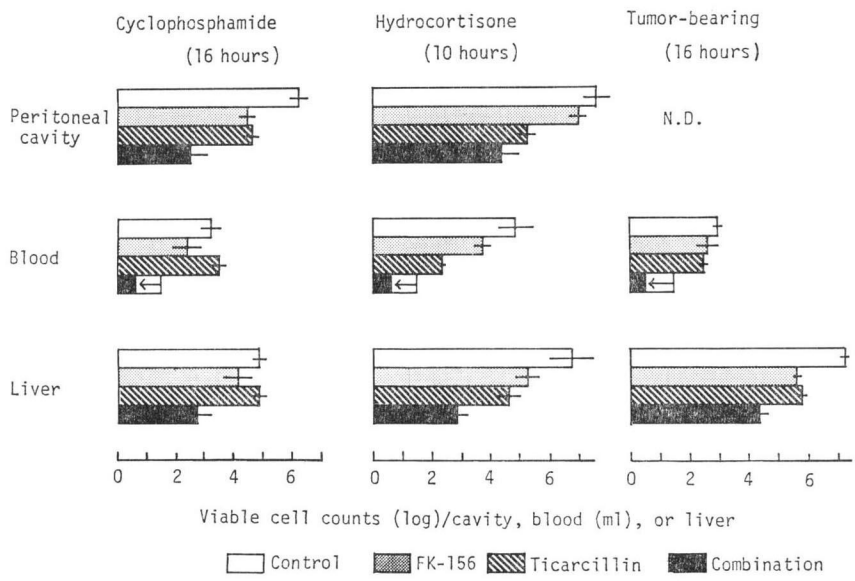
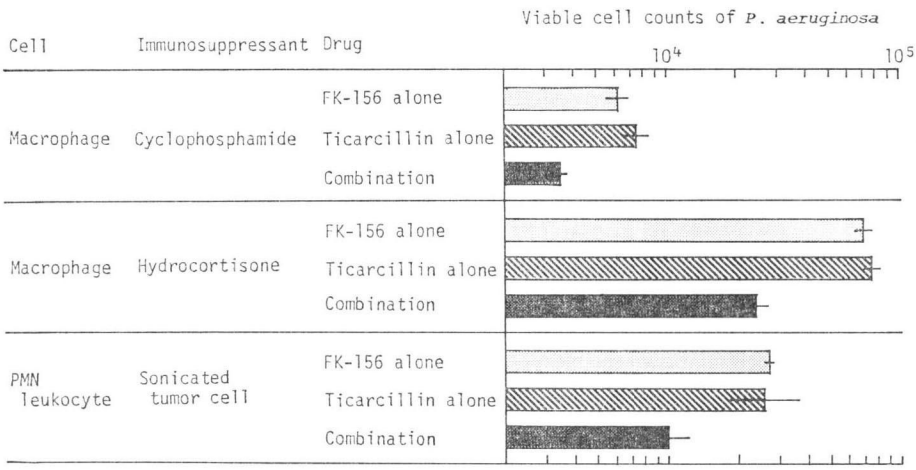


Fig. 3. Synergism of FK-156 and ticarcillin on phagocytosis and killing of *P. aeruginosa* by peritoneal PMN leukocyte and macrophage of immunosuppressed mice.



When peritoneal PMN leukocytes obtained from normal mice were incubated *in vitro* with tumor cells sonicated and FK-156 or ticarcillin, the viable cell counts were significantly lower when the drugs were used in combination than when used singly.

Discussion

In the present study, cyclophosphamide, hydrocortisone, carrageenan, mitomycin C and implantation of tumor cells caused immunosuppression in mice. These immunosuppressants have been shown to subvert many factors participating in the host defense mechanisms such as PMN leukocytes, macrophage and lymphocyte; exudate response; phagocytic and killing activities; chemotaxis of phagocytes; and RES function. There have been various reports on experimental infections in animal models immunosuppressed with cyclophosphamide or hydrocortisone[4~9]. In our study the susceptibility to microbial infection was markedly higher in immunosuppressed mice than in normal mice and the therapeutic effect of antibiotics on microbial infection was significantly lower than in normal mice. These results observed on microbial infections in immunosuppressed mice parallel the increased vulnerability of defense mechanisms in immunocompromised patients. We investigated the possibility of using immunostimulants to enhance the efficacy of chemotherapeutics in immunocompromised patients in whom good therapeutic effect can not be obtained by antibiotics alone. The concept of immune stimulation by immunostimulants is largely still in the experimental stage. The experiments being performed, however, have become more numerous and meaningful over the last few years. Muramyl dipeptide and its analogues,[10~14] BCG[15,16], *Corynebacterium parvum*[17], krestin[18], glucan[19,20], ubiquinon-8[21], azimexon[22] and phospholipid[23] have been reported to enhance host resistance to various microbial infections in animals. FK-156, a new immunostimulant, is a lactoyl tetrapeptide obtained from *Streptomyces olivaceogriseus* sp. nov. in our Research Laboratories. FK-156 enhanced host resistance to infections due to extracellular and facultative intracellular parasites in both immunosuppressed and normal mice, although it has no direct antibacterial or antifungal activity *in vitro*. The mechanisms of the protective effect of the drug on microbial infection are elucidated in the following paper[24]. FK-156 restored depressed defense mechanisms against microbial invasion in immunosuppressed mice by 1) increasing peritoneal PMN, macrophage and lymphocyte counts, 2) stimulating exudate response, and the chemotactic, phagocytic and killing activities of macrophages and PMN, and 3) enhancing RES function. Under conditions in which antibiotics and FK156 alone were therapeutically ineffective, the protective effect of ticarcillin was markedly enhanced by combined therapy with FK-156. The protective synergism

of ticarcillin and FK-156 against infection was considered to be induced both by restoration of the defense mechanism by FK-156 and by the synergistic bacterial effect of ticarcillin and FK-156-activated phagocytes. Therefore, these results suggest that antibiotic-FK-156 therapy may be useful in treating microbial infections while hosts are temporarily immunosuppressed, as is the case in cancer chemotherapy and radiation injury. However, before clinical application, it is necessary to confirm that FK-156 is nontoxic at effective dose levels.

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