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

ENHANCEMENT OF HOST RESISTANCE TO MICROBIAL INFECTION IN MICE

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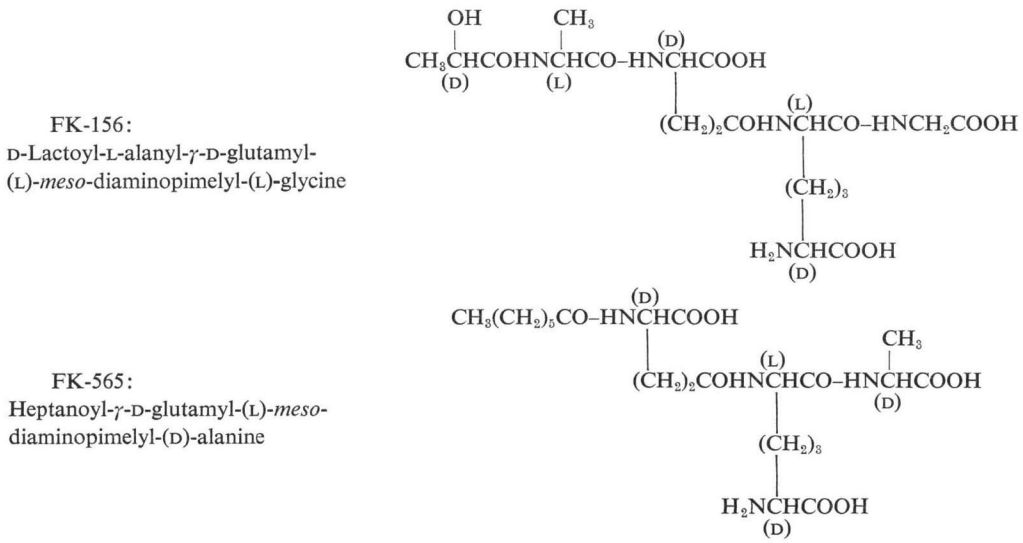
The protective effect of an immunoactive peptide, D-lactoyl-L-alanyl-γ-D-glutamyl-(L)-*meso*-diaminopimelyl-(L)-glycine (FK-156) and a related compound, heptanoyl-γ-D-glutamyl-(L)-*meso*-diaminopimelyl-(D)-alanine (FK-565) was determined in mice with various kinds of microbial infections. FK-156 and FK-565 were given to mice either subcutaneously or orally before challenge. The drugs enhanced significantly the defense of mice against acute systemic infections induced by various extracellular and facultative intracellular organisms, and subcutaneous abscess by *Staphylococcus aureus*. The protective effect of these drugs against *Escherichia coli* infection differed considerably depending on the route of administration; FK-156 was only effective by the parenteral route; however, FK-565 was effective by both parenteral and oral routes. After subcutaneous dosing with FK-156, the enhancement of host defense of mice against *E. coli* infection was more rapid than against Listeria infection. The enhancing effects of FK-156 and FK-565 on host defense of mice against pseudomonal infection was more potent than other immunoactive drugs.

Various microbial products such as BCG[1], *Corynebacterium parvum*[2], endotoxin[3], glucan[4], krestin[5] and phospholipids[6] have been shown to enhance host resistance to microbial infection in

Fig. 1. Chemical structure of FK-156 and FK-565.



experimental animals. In addition, the synthetic compounds, azimexon[7], muramyl dipeptide and its analogs[8~10] have also been reported to induce similar resistance-enhancing effects. Recently, in the course of extensive screening for immunostimulants in the Fujisawa Research Laboratories, a lactoyl tetrapeptide (FK-156) was isolated from cultures of *Streptomyces olivaceogriseus* sp. nov.[11~15] A related peptide, FK-565 was also synthesized (Fig. 1)[16].

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. The following drugs were used as reference drugs: N-CWS (cell wall skelton of *Nocardia rubra*, Fujisawa Research Laboratories), levamisole (Sigma Chemical Company), krestin (Kureha Co., Ltd.) and picibanil (Chugai Pharmaceutical Co., Ltd.). Muramyl dipeptide (MDP) and lauroyl tetrapeptide (LTP) were synthesized in the Fujisawa Research Laboratories. FK-156 and FK-565 were dissolved in 0.85% saline at various concentrations, and given to mice at administration schedules described in the attached Figures and Tables.

Bacterial Strains

Clinical isolates of various species of bacteria were used.

Protection Testing in Mice

Male ICR strain mice aged 4 weeks, unless otherwise specified, were used in groups of 10. The mice were inoculated intraperitoneally and intravenously with bacterial suspensions of 0.5 ml, each containing inoculum sizes in a range from LD_{50} to the minimum lethal dose. In the case of salmonellosis, *dd*Y strain mice (4 weeks old) were used. The mice were fasted overnight and challenged orally with *Salmonella enteritidis* strain FP233 at 10 cfu/mouse, 3 times at 6-hour intervals. The protective effect was estimated by the number of mice surviving after 4 to 14 days of observation. For subcutaneous staphylococcal abscess, the mice were inoculated subcutaneously with 2.0×10^7 cfu/site on the lower back for the FK-156- and 3.4×10^7 cfu/site for the FK-565-treated mice. The skin of the inoculation site was excised 5 days after challenge and homogenized in saline with a Polytron. Viable cell counts were determined by plating on agar and counting colony forming units (cfu).

Results

Acute Systemic Infection

The effect of FK-156 and FK-565 on host-defense ability of mice against microbial infection was evaluated. As shown in Table 2, survival rates in animals with microbial infection due to extracellular organisms, such as *Escherichia coli* strain 22, *Klebsiella pneumoniae* strain 57, *Serratia marcescens* strain 32 and *Pseudomonas aeruginosa* strain 97 were 70 to 100% when FK-156 was given in a single subcutaneous dose of 1 mg/kg 4 days or 1 day before challenge, and 40 to 80% when FK-565 was given orally in a dose of 0.1 mg/kg 6, 5, 4 and 1 day before challenge. In addition, both peptides, FK-156 and FK-565 were effective against microbial infection due to facultative intracellular parasites such as Listeria and Salmonella. The survival rate of mice given FK-156 was 40% for *Listeria monocytogenes* strain FP566 and 50% and 70% respectively for intravenous and oral challenge with *Salmonella enteritidis* strain FP233. The survival rates of mice given FK-565 were 80 to 90% for these 2 bacterial infections, whereas survival rates for mice infected with *Candida albicans* strain FP-633 were 30% for FK-156 and 80% for FK-565. The host defense ability of mice given the peptides was enhanced against all strains tested, although the protective effect varied among the challenge organisms.

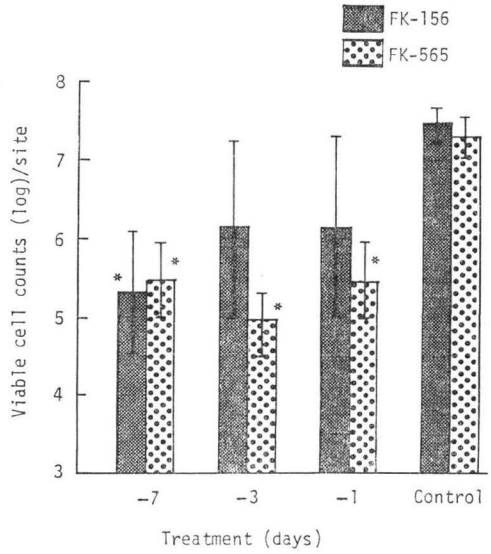
Table 1. Effect of dosing routes of FK-156 and FK-565 on *E. coli* infection in mice.

Route	% Survival			
	FK-156		FK-565	
	1 mg/kg	0.1 mg/kg	1 mg/kg	0.1 mg/kg
i.p.	90	40	100	70
i.v.	60	40	80	40
s.c.	50	30	70	50
p.o.	10	10	90	30
Control	0		20	

FK-156 and FK-565 were given intraperitoneally, intravenously and subcutaneously, 4 days before challenge and orally 6, 5 4 and 1 day before challenge with *E. coli* No. 22.

Fig. 2. Effect of FK-156 and FK-565 on viable cell counts in staphylococcal abscess in mice.

FK-156 was given subcutaneously in a dose of 10 mg/kg, and FK-565 orally in a dose of 1 mg/kg. Viable cell counts were determined 5 days after subcutaneous inoculation with *S. aureus*. $*P<0.05$



Subcutaneous Abscess Due to *Staphylococcus aureus*

A single dose of FK-156 (10 mg/kg) or FK-565 (1 mg/kg) was given to mice on different days before subcutaneous challenge with *S. aureus* strain 169, and the viable cell counts in the resultant abscess were determined. As shown in Fig. 2, the viable cell counts (5.30 ± 0.79, mean logarithm) in the staphylococcal abscess significantly decreased as compared with those (7.48 ± 0.20) in the non-treated mice, when mice were dosed with FK-156 7 days before challenge. A similar decrease of the viable cell count was also seen when the drug was given 1 or 3 days before challenge. A significant decrease of viable cell count at the site of abscess was also observed when FK-565 was given orally to mice at 7 or 3 days or 1 day before challenge.

Effect of Various Routes of Dosing

The effects of different routes of dosing were compared in mice with *E. coli* infection (Table 1). A single parenteral dose of 0.1 or 1 mg/kg of the drugs was given to mice 4 days before challenge, or the

Table 2. Effect of FK-156 and FK-565 on host defense of mice against various microbial infections.

Organism	(Route)	% Survival			
		FK-156[a]		FK-565[b]	
		1 mg/kg/day	Control	0.1 mg/kg/day	Control
E. coli No. 22	(i.p.)	90#	0	60	10
K. pneumoniae No. 57	(i.p.)	70	0	40	20
S. marcescens No. 32	(i.p.)	80	20	80	20
P. aeruginosa No. 97	(i.p.)	100	10	70	10
C. albicans FP633	(i.v.)	30	0	80	40
L. monocytogenes FP566	(i.p.)	40#	0	90	0
S. enteritidis FP233	(i.v.)	50#	10	80	20
S. enteritidis FP233	(p.o.)	70#	40	80	40

[a] FK156 was given subcutaneously 4 days# or 1 day before challenge.

[b] FK565 was given orally 6, 5, 4 and 1 day before challenge.

drugs of 0.1 or 1 mg/kg/day were given orally to mice 4 times (at 6, 5, 4 days and 1 day) before challenge. Intraperitoneally administered FK-156 (1 mg/kg) was the most effective against *E. coli* infection, followed by the intravenous and subcutaneous routes, whereas oral dosing 4 times before challenge was not effective. With 0.1 mg/kg, no marked difference was found among the parenteral routes used. FK-565 (1 mg/kg) by the intraperitoneal route was also the most effective, followed by the oral, intravenous and subcutaneous routes. With 0.1 mg/kg, the intraperitoneal route was also more effective than the other routes.

Time-dependence in Protective Effect of FK-156 on *E. coli* and Listeria Infection

A single intraperitoneal dose of FK-156 (1 mg/kg) was given to mice at different intervals before challenge with *E. coli* strain 22 or *L. monocytogenes* strain FP566.

Table 3 shows that the resistance of mice to *E. coli* infection was rapidly enhanced and persisted well. The survival rate was as high as 40% when FK-156 was given 7 days before challenge and as high as 90% when the drug was given on the day before challenge. However, as long as 4 days was needed to enhance the protective ability of mice against Listeria infection.

Table 3. Time-dependence in protective effect of FK-156 on *E. coli* and *L. monocytogenes* infection in mice.

Organism	Treatment (Day)	Survival (%)
E. coli No. 22	−7	40
	−4	90
	−1	90
	Control	0
L. monocytogenes FP566	−7	50
	−4	90
	−1	0
	Control	10

Mice were given intraperitoneally 1 mg/kg of FK-156, and inoculated intraperitoneally 4.8×10^7 cfu/mouse with *E. coli* No. 22 and 7.5×10^7 cfu/mouse of *L. monocytogenes* FP566.

Table 4. Effect of FK-156, FK-565 and some immunostimulants on defensive ability of mice against pseudomonal infection.

Immunostimulant	% Survival			
	Treatment (Day)			
	−7		−1	
	1 mg/kg	0.1 mg/kg	1 mg/kg	0.1 mg/kg
FK-156	70	60	100	70
FK-565	90	50	80	100
MDP	20	20	50	20
N-CWS	70	50	50	20
Krestin	40	40	20	10
Picibanil	40	30	20	20
Levamisole	60	50	10	0
LTP	20	50	30	10
Control	10			

These immunostimulants were subcutaneously 7 or 1 day before challenge with *P. aeruginosa*.

Comparison with Other Immunostimulants in Pseudomonal Infection

The test drugs were given in a single subcutaneous dose of 1 or 0.1 mg/kg to mice 7 days or 1 day before challenge with *P. aeruginosa*.

The protective effect and onset of effect of immunostimulants varied by drug and time of dosing. However, the protective effect of both FK-156 and FK-565 was the most potent of all the test drugs after both dosing times and did not vary in time of onset.

Therefore, the defensive ability of mice against pseudomonal infection was rapidly enhanced and persisted until the end of the study.

Discussion

In spite of the advent of potent new antibiotics, we still face many problems in the chemotherapy of

infection in immunocompromised patients. Therapeutic attempts to enhance overall host resistance to infection by restoring impaired defense mechanisms have been carried out in many experimental models. Various microbial products such as BCG[1], *Corynebacterium parvum*[2], endotoxin[3], glucan[4], krestin[5], phospholipid[6] have been shown to enhance host resistance to infection. However, most of these substances are macromolecular, insoluble, pyrogenic and not well defined chemically. Recently, a synthetic compound, MDP has been shown to possess the minimal active structure essential for eliciting the effect of the whole mycobacterial cell contained in Freund's complete adjuvant.[17~19] MDP and its analogues have also been shown to increase nonspecific resistance against infection[8~10].

FK-156 used in this study is an acyl peptide, composed of D-lactic acid and four amino acids, L-alanine, D-glutamic acid, α,ε-*meso*-diaminopimelic acid and glycine. It was first extracted from the fermentation broth of *S. olivaceogriseus*[11~15]. However, the compound has been successfully synthesized chemically. Its analogue, FK-565, is composed of heptanoic acid and the three amino acids, D-glutamic acid, α,ε-*meso*-diaminopimelic acid and D-alanine[16].

The host defense ability of mice was enhanced markedly when FK-156 and FK-565 were given subcutaneously or orally before systemic infection induced by extracellular organisms such as *E. coli, K. pneumoniae, S. marcescens* and *P. aeruginosa* and facultative intracellular parasites such as Candida, Listeria and Salmonella. FK-156 and FK-565 were also effective against local infection *e.g.* staphylococcal abscess, as well as systemic infection. FK-156 was markedly active against *E. coli* infection, when given parenterally (intraperitoneal, intravenous and subcutaneous) but not by the oral route. It is therefore of note that both small oral and parenteral doses of its analogue, FK-565 significantly enhanced host resistance. The effect of FK-156 on host resistance depended markedly on pretreatment time, *i.e.*, one day in the case of *E. coli* which is mainly phagocytosed and killed by PMN leucocytes, and 4 days in the case of *L. monocytogenes* by macrophages.

The protective effect of these immunostimulants was affected by the route, time and dosing and the inoculum size of the organisms. KIERZENBAUM and FERRARESI[20] have shown that the protective effect of MDP against *Trypanosoma cruzi* infection depended on treatment time and HUMPHRES *et al.*[21], reported that the increase of host resistance induced by MDP depended on the route of administration and species of challenge organisms. The protection elicited by the test drugs given 7 days and 1 day before Pseudomonas challenge shows that the survivability of the host treated with FK-156 and FK-565 was enhanced rapidly and maintained until the end of the study. It is reasonable therefore, that FK-156 and FK-565 were effective against local persistent infections such as staphylococcal abscess, which suggests they might be efficacious in other persistent infections.

References

1) SHER, N. A.; S. D. CHAPARAS, L. E. GREENBENG & S. BERNARD: Effect of BCG, *Corynebacterium parvum*, and methanol-extraction residue in the reduction of mortality from *Staphylococcus aureus* and *Candida albicans* infection in immunosuppressed mice. Infect. Immun. 12: 1325~1330, 1975
2) BERD, D.: Effects of *Corynebacterium parvum* on immunity. Pharmacol. Ther. A2: 373~395, 1978
3) DUBOS, R. & R. W. SCHAEDLER: Reversible changes in the susceptibility of mice to bacterial infections. J. Exp. Med. 104: 53~65, 1956
4) REYNOLDS, J. A.; M. D. KASTELLO, D. G. HANINGTON, C. L. CRABBS, C. J. PERTERS, J. V. JEMSKI, G. H. SCOTT & N. R. D. LUZIO: Glucan-induced enhancement of host resistance to selected infectious diseases. Infect. Immun. 30: 51~57, 1980
5) MAYER, P. & J. DREWS: The effect of a protein-bound polysaccharide from *Coriolus versicolor* on immunological parameters and experimental infections in mice. Infection 8: 13~21, 1980
6) FANVE, R. M. & B. HEVIN: Immunostimulation with bacterial phospholipid extracts. Proc. Nat. Acad. Sci. 71: 573, 1974
7) BICKER, V.; A. E. ZIEGLER & G. HEBOLD: Investigations in mice on the potentiation of resistance to infections by an new immunostimulant compound. J. Infect. Dis. 139: 389~395, 1979
8) CHEDID, L.; M. PARANT, F. PARANT, P. LEFRANCIER, J. CHOAY & E. LEDERER: Enhancement of nonspecific immunity to *Klebsiella pneumoniae* infection by a synthetic immunoadjuvant (*N*-acetyl muramyl-L-alanyl-D-isoglutamine) and several analogues. Proc. Nat. Acad. Sci. 74: 2089~2093, 1977

9) PARANT, M.; F. PARANT & L. CHEDID: Enhancement of the Neonate's nonspecific immunity to Klebsiella infection by muramyl dipeptide, a synthetic immunoadjuvant. Proc. Nat. Acad. Sci. 75: 3395~3399, 1978
10) PARANT, M.; F. PARANT, L. CHEDID, M. LEVEL, P. LEFRANCIER, J. CHOAY & E. LEDERE: Immunostimulant activities of a lipophilic muramyl dipeptide derivatives and of desmuramyl peptidelipid analogs. Infect. Immun. 27: 826~831, 1980
11) GOTOH, T.; K. NAKAHARA, M. IWAMI, H. AOKI & H. IMANAKA: Studies on a new immunoactive peptide, FK-156. I. Taxonomy of the producing strains. J. Antibiotics 35: 1280~1285, 1982
12) GOTOH, T.; K. NAKAHARA, T. NISHIURA, M. HASHIMOTO, T. KINO, Y. KURODA, M. OKUHARA, M. KOHSAKA, H. AOKI & H. IMANAKA: Studies on a new immunoactive peptide, FK-156. II. Fermentation, extraction and chemical and biological characterization. J. Antibiotics 35: 1286~1292, 1982
13) KAWAI, Y.; K. NAKAHARA, T. GOTOH, I. UCHIDA, H. TANAKA & H. IMANAKA: Studies on a new immunoactive peptide, FK-156. III. Structure elucidation. J. Antibiotics 35: 1293~1299, 1982
14) HEMMI, K.; M. ARATANI, H. TAKENO, S. OKADA, Y. MIYAZAKI, O. NAKAGUCHI, Y. KITAURA & M. HASHIMOTO: Studies on a new immunoactive peptide, FK-156. IV. Synthesis of FK-156 and its geometric isomer. J. Antibiotics 35: 1300~1311, 1982
15) HEMMI, K.; H. TAKENO, S. OKADA, O. NAKAGUCHI, Y. KITAURA & M. HASHIMOTO: Total synthesis of FK-156 isolated from a Streptomyces as an immunostimulating peptide: Application of a novel copper chelate amino protection. J. Am. Chem. Soc. 103: 7026~7028, 1981
16) KITAURA, Y.; H. TAKENO, M. ARATANI, S. OKADA, S. YONISHI, K. HEMMI, O. NAKAGUCHI & M. HASHIMOTO: Synthesis and RES stimulating activity of bacterial cell-wall peptidoglycan peptides related to FK-156. Experimentia 38: 1101~1103, 1982
17) CHEDID, L. M.; F. AUDIBERT, P. LEFRANCIER, J. CHOAY & E. LEDERER: Modulation of the immune response by a synthetic adjuvant and analogs. Proc. Nat. Acad. Sci. 73: 2472~2475, 1976
18) ELLOUZ, F.; A. ADAM, R. CIORBARU & E. LEDERER: Minimal structural requirements for adjuvant activity of bacterial peptidoglycan derivatives. Biochem. Biophys. Res. Commun. 59: 1317~1325, 1974
19) KOTANI, S.; Y. WATANABE, F. KINOSHITA, T. SHIMONO, I. MORISAKI, T. SHIBA, S. KUSUMOTO, Y. TARUMI & K. IKENAKA: Immunoadjuvant activities of synthetic *N*-acetylmuramylpeptides or -aminoacids. Biken J. 18: 105~111, 1975
20) KIERSZENBAUM, F. & R. W. FERRARESI: Enhancement of host resistance against *Trypanosoma cruzi* infection by the immunoregulatory agent muramyl dipeptide. Infect. Immun. 25: 273~278, 1979
21) HUMPHRES, C.; P. R. HENIKA, R. W. FERRARESI & J. L. KRAHENBUHL: Effect of treatment with muramyl dipeptide and certain of its analogs on resistance to *Listeria monocytogenes* in mice. Infect. Immun. 30: 462~466, 1980