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

ENHANCEMENT OF HOST DEFENSE MECHANISMS AGAINST INFECTION

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We investigated the effect of immunoactive peptides, FK-156 and FK-565 on host defense mechanisms against microbial invasion. It was shown that these drugs given to normal mice increased the counts of phagocytes in both peripheral blood and peritoneal cavity, and enhanced the chemotactic, phagocytic and killing activities of peritoneal macrophages and polymorphonuclear leukocytes, and stimulated the phagocytic function of the reticuloendothelial system. Enhanced host resistance to microbial infection by these immunoactive peptides might be induced by both increase in counts and enhancement of functions of phagocytes. FK-156 restored decreased counts and functions of phagocytes in mice immunosuppressed by cyclophosphamide, hydrocortisone or tumor. These findings suggest that these immunoactive peptides could be applied to prevent intractable infection in immunocompromised hosts.

We reported that FK-156, an immunoactive peptide isolated from culture filtrates of *Streptomyces olivaceogriseus* sp. nov.[1~4], and its synthetic analogue FK-565 afforded resistance to microbial infections due to facultative intracellular bacteria, extracellular pathogens and fungi in normal and immunosuppressed mice[5,6]. It is well known that phagocytes play an important role in host defense mechanisms and it has been reported that such agents as Bacille Calmette Guerin (BCG), lipopolysaccharide (LPS), *Nocardia rubra* cell wall skeleton (N-CWS) and muramyldipeptide (MDP) activate phagocytic functions[7~10]. In this study, we investigated the effects of FK-156 and FK-565 on the counts and functions of phagocytes in both normal and immunosuppressed mice to elucidate the mechanism of their action on the enhancement of resistance to infection.

Materials and Methods

Animals

Four-week old male mice of ICR and *dd*Y strains were used.

Microorganisms

Pseudomonas aeruginosa strain 97, *Listeria monocytogenes* strain FP566 and *Candida albicans* strain FP633 were clinical isolates. These strains were grown respectively for 20 hours on Trypticase Soy Agar, Brain Heart Infusion Agar and Sabouraud dextrose agar slants, and were suspended in HANKS' balanced salt solution (HBSS) for experiments.

Immunostimulants

FK-156 and FK-565 were prepared in the Fujisawa Research Laboratories, Osaka, Japan.

Immunosuppressants

Cyclophosphamide (Endoxan, Shionogi & Co., Ltd.), hydrocortisone (Nakarai Chemical Ltd.), sarcoma 180 in ascites form and sonicated sarcoma 180 ascites were used. Cell-free supernatant of sonicated sarcoma 180 was prepared as described by PIKE and SNYDERMAN[11].

Immunosuppressed Mice

Cyclophosphamide was given in a single intraperitoneal dose of 200 mg/kg 4 days before experiment. Hydrocortisone was given in subcutaneous doses of 50 mg/kg once a day for 4 days before experiment. Sarcoma 180 tumor cells were implanted intraperitoneally at a concentration of 6×10^6 cells per mouse 8 days before experiment. When the phagocytic functions of tumor-bearing mice was studied *in vitro*, phagocytes from normal mice were treated with a 10% supernatant of sonicated tumor cells.

Peripheral and Peritoneal Leukocyte Counts

Blood was collected from the heart of normal and immunosuppressed mice anesthetized with chloroform and the leukocytes were counted with a hematology analyzer HA/4 (Becton & Dickens Co.). Peritoneal cells were collected from the peritoneal cavity of the same mouse and counted with a hemocytometer. Differential counts of these leukocytes were done in preparations made with a sedimentation apparatus, the cells being sedimented on a microscope slide, fixed in ethanol, and stained with Giemsa stain.

Preparation of Phagocytes

Peritoneal macrophage suspension was prepared by the method described by BJORNSON[12]. Peritoneal polymorphonuclear leukocyte (PMN) suspension was obtained from mice 3 hours after intraperitoneal injection of 2.0 ml of 0.5% glycogen solution. The purity of each suspension was not less than 90%.

In Vitro Chemotaxis

PMN and macrophage chemotaxis were quantified by a minor modification of BOYDEN's method[13] using polycarbonate filters and Blind Well Chambers (Bio-Rad Inc.). 0.2 ml of LPS-activated serum was used as a chemoattractant and was placed in the lower compartment, and incubated at 37°C for 1 hour and 4 hours respectively for PMN and macrophages. Chemotaxis was quantified by counting and averaging the number of leukocytes migrated per 10 microscopic fields (0.0625 mm^2) and was expressed as the chemotactic index.

Phagocytosis and Killing

0.35 ml of phagocyte suspension, 0.05 ml of autologous serum and 0.1 ml of bacterial suspension were placed in a siliconized glass tube with a rubber stopper. This mixture contained about 8×10^6 phagocytes, 10% fresh mouse serum and 1×10^5 cfu of bacteria per ml in HBSS. The mixture was incubated at 37°C for 2 hours under continuous rotation (4 rpm). After destruction of phagocytes with 0.1% sodium dodecyl sulfate, the total viable bacterial counts were determined by plating on agar and determining colony forming units.

Carbon Clearance

The carbon clearance test was carried out by the method of BIOZZI *et al.*[14] to determine the function of the reticuloendothelial system (RES). The phagocytic index (K value) was calculated by the following formula:

$$K=(\log C_1-\log C_2)/T_2-T_1$$

where C_1 and C_2 are blood carbon concentrations at times T_1 and T_2 after injection of carbon particles.

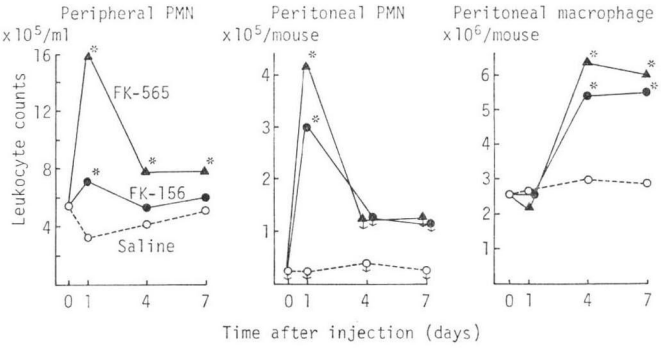
Results

Normal Mice

Phagocytic Counts

Fig. 1 shows the counts of phagocytes in the peripheral blood and peritoneal cavity of normal mice. FK-156 and FK-565 significantly increased peripheral and peritoneal PMN counts and peritoneal macro-

Fig. 1. Peripheral and peritoneal leukocyte counts in FK-156- and FK-565-treated mice.
Mice were used in groups of 5. The arrow show counts less than values.
* Significantly different from saline-treated mice ($P<0.05$)



phage counts 1 day and 4 days respectively after a single subcutaneous injection at a dose of 1 mg/kg. FK-565 was more effective than FK-156 in all cases. Peripheral monocyte counts also tended to increase after injection (data not shown).

Chemotaxis

Table 1 shows the chemotactic responses of PMN and macrophages toward LPS-activated serum. PMN and macrophages were obtained from mice 4 days after subcutaneous and intraperitoneal injection of 1 mg/kg of the drug. The chemotactic activities of PMN from FK-156- and FK-565-treated mice were enhanced 5.6 and 5.2 times respectively when compared with that of control PMN from saline-treated mice. Also, the chemotactic activity of macrophages from mice treated with either drug was enhanced about 11 times when compared to control macrophages. These results show that both drugs enhanced the response of phagocytes to a chemotactic factor.

Table 1. *In vitro* chemotactic activity of peritoneal PMN and macrophages from FK-156- and FK-565-treated mice.

Drug	Chemotactic index	
	PMN	Macrophages
FK-156	44.8±8.4[a] (5.6)[b]	52.5±6.5[a] (11.4)
FK-565	41.4±7.6[a] (5.2)	51.7±7.3[a] (11.2)
Saline	8.0±4.7	4.6±1.7

PMN and macrophages were obtained from mice 4 days after respective subcutaneous and intraperitoneal injections of 1 mg/kg of the drugs.

[a] Significantly different from control phagocytes ($P<0.01$)

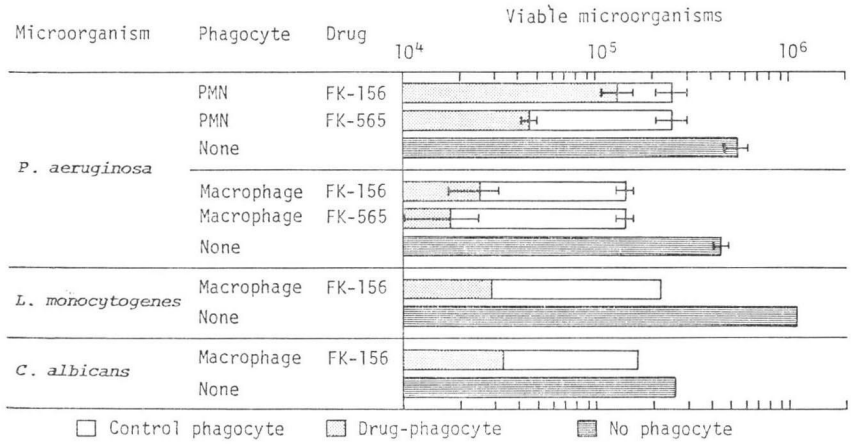
[b] Ratio of chemotactic index of drug-treated mice to that of control mice

Phagocytosis and Killing

Fig. 2 shows the *in vitro* phagocytic and killing activity of peritoneal PMN and macrophages from saline-treated and drug-treated mice. Extracellular bacteria, *P. aeruginosa*, facultative intracellular bacteria, *L. monocytogenes*, and fungus, *C. albicans* were used for this experiment. Viable counts of *P. aeruginosa* after incubation with PMN from FK-156- and FK-565-treated mice decreased by 1.5×10^5 and 4.3×10^4 cfu/ml respectively compared to 2.6×10^5 cfu/ml in the case of PMN from control mice. After incubation with macrophages from FK-156- and FK-565-treated mice, viable counts of *P. aeruginosa* decreased by 2.5×10^4 and 1.8×10^4 cfu/ml respectively compared to 1.5×10^5 cfu/ml in the case of control macrophages. Similarly, viable counts of *L. monocytogenes* and *C. albicans* were lower after incubation with macrophages from drug-treated mice compared to those from control mice. Namely, FK-156 and FK-565 enhanced the phagocytic and killing activity of mouse peritoneal phagocytes against extracellular bacteria, facultative intracellular bacteria and fungi.

Fig. 2. Phagocytosis and killing of microorganisms by peritoneal PMN and macrophages from FK-156- and FK-565-treated mice.

Phagocytes were obtained as described in Table 1. Experiments using *P. aeruginosa* were in duplicate; the others were single experiments.



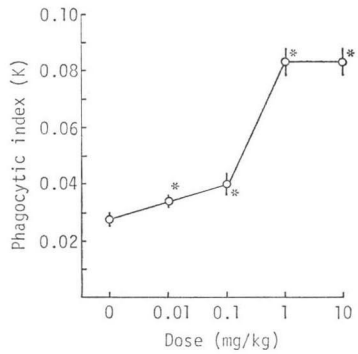
Carbon Clearance

The phagocytic function of the RES in mice was investigated by the carbon clearance method. Fig. 3 shows the carbon clearance rates in mice 24 hours after intraperitoneal injection of several doses of FK-156. The phagocytic index in FK-156-treated mice increased dose-dependently at doses of 0.01 to 1 mg/kg and the indexes at doses of 1 and 10 mg/kg were 3 times higher than in the non-treated control mice. Hence, FK-156 stimulated the phagocytic function of the RES.

Fig. 3. Clearance rates of carbon from blood-stream in FK-156-treated mice.

Cabon clearance test was performed 24 hours after intraperitoneal injection of FK156 (n=10).

* Significantly different from control mice ($P<0.05$).



Immunosuppressed Mice

The effect of FK-156 on phagocytes was also studied in mice immunosuppressed by cyclophosphamide, hydrocortisone or tumor. FK-156 was given to immunosuppressed mice twice in subcutaneous doses of 1 mg/kg 3 days and 1 day before experiment.

Phagocytic Counts

Fig. 4 shows the peritoneal phagocytic counts in cyclophosphamide- and hydrocortisone-treated mice. In the cyclophosphamide-treated mice the main population of peritoneal cells, the macrophages, significantly increased by two-fold after treatment with FK-156. Similarly in the hydrocortisone-treated mice, PMN counts increased by more than 10-fold, so that by these two actions, the peritoneal phagocytic counts were restored to normal levels.

Chemotaxis

Table 2 shows the *in vitro* chemotaxis of phagocytes from immunosuppressed mice. The chemotactic activity of peritoneal PMN from hydrocortisone-treated mice, with or without FK-156 treatment,

Fig. 4. Effect of FK-156 on peritoneal PMN and macrophage counts in immunosuppressed mice. Mice were used in groups of 5.

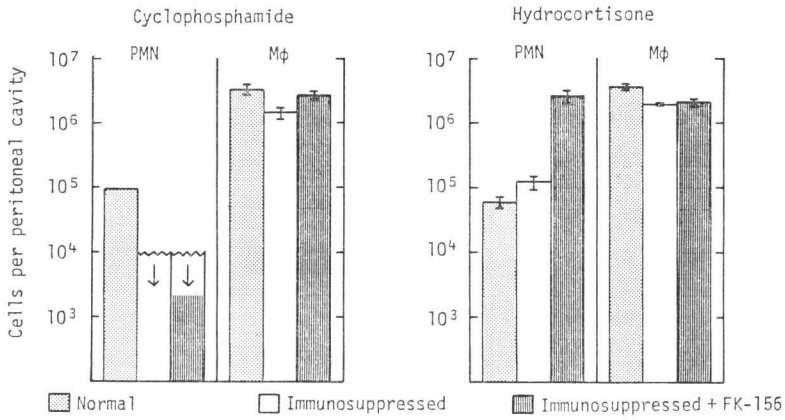


Table 2. Effect of FK-156 on *in vitro* chemotaxis of PMN and macrophages from immunosuppressed mice.

Phagocyte	Immunosuppressant	Drug	Chemotactic index	% Inhibition[a]	Stimulation[b] index
RMN	None (Normal)	None	169.0±17.0		
	Hydrocortisone	None	12.0± 5.0	93	
	Hydrocortisone	FK-156	50.0±28.0	70	4.2
	None (Normal)	None	18.2± 2.9		
	Sonicated tumor cell	None	5.2± 2.1	71	
	Sonicated tumor cell	FK-156	14.7± 2.5	19	2.8
Macrophage	None (Normal)	None	32.1± 9.9		
	Sonicated tumor cell	None	5.0± 1.8	84	
	Sonicated tumor cell	FK-156	27.2± 8.0	15	5.4

[a] Percentage of inhibition=100−(Chemotactic index of immunosuppressed mice/normal mice)×100.
[b] Stimulation index=Chemotactic index of FK-156-treated immunosuppressed mice/immunosuppressed mice.

was compared to that of normal mice. Hydrocortisone markedly depressed the chemotactic activity of PMN (93% inhibition), but FK-156 restored it to some extent (stimulation index: 4.2). The effect of FK-156 on the chemotactic activity in tumor-bearing mice was studied in a complete *in vitro* system. That is, chemotaxis was measured under the conditions that phagocytes from normal mice were treated with the supernatant of sonicated sarcoma 180 tumor cells and FK-156 *in vitro*. Supernatant of sonicated sarcoma 180 depressed the chemotactic activity of PMN and macrophages by 71% and 84%, respectively. However, the addition of FK-156 at the concentration of 10 μg/ml into the incubation system restored the chemotactic activity of PMN and macrophages to nearly normal levels (stimulation indexes were 2.8 and 5.4, respectively).

Phagocytosis and Killing

Fig. 5 shows the phagocytic and killing activity of phagocytes from immunosuppressed mice. Viable counts of *P. aeruginosa* after incubation with PMN from hydrocortisone-treated and normal mice were similar. Thus, hydrocortisone did not affect the phagocytic and killing activity of PMN. However, when FK-156 was given to hydrocortisone-treated mice, the PMN significantly decreased the viable counts indicating that FK-156 enhanced the phagocytic and killing activity of PMN from hydrocortisone-

Fig. 5. Effect of FK-156 on phagocytosis and killing of *P. aeruginosa* by peritoneal PMN and macrophages from immunosuppressed mice.

Each experiment was performed in duplicate.

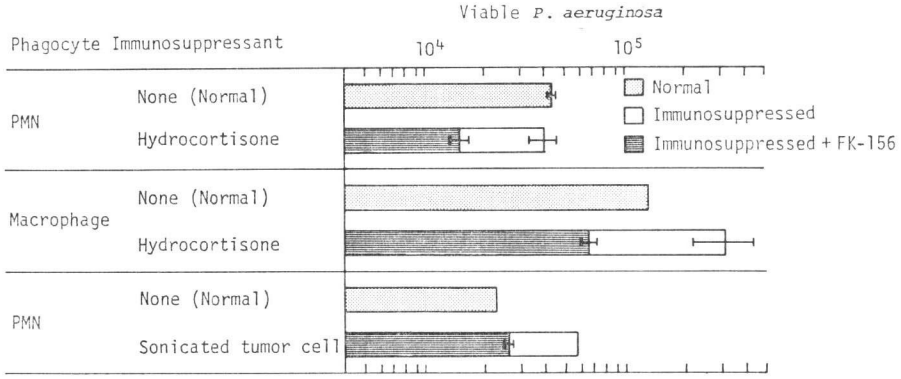
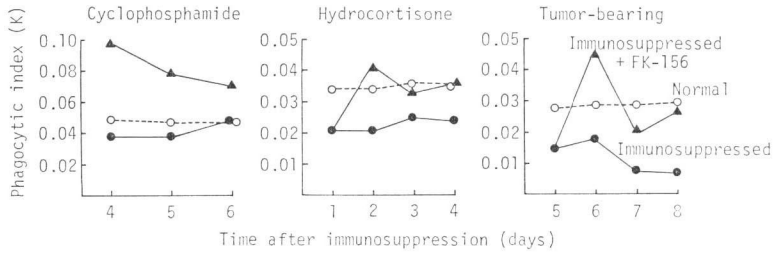


Fig. 6. Effect of FK-156 on clearance rates of carbon from blood-stream in immunosuppressed mice. Mice were used in groups of 10.



treated mice. Hydrocortisone depressed the phagocytic and killing activity of macrophages, but FK-156 restored this depression. Similarly, *in vitro* treatment with the supernatant of sonicated tumor cells depressed the phagocytic and killing activity of PMN, but the addition of FK-156 restored this depression.

Carbon Clearance

Fig. 6 shows the clearance rates of carbon from the blood-stream in 3 kinds of immunosuppressed mice. The phagocytic index in each immunosuppressed mice was lower than in normal mice. However, FK-156 enhanced carbon clearance beyond the normal level in cyclophosphamide-treated mice and restored it to the normal level in hydrocortisone-treated and tumor-bearing mice.

Discussion

We reported that FK-156 and FK-565, immunoactive peptides, afforded resistance to infection in mice[5,6]. In this study we investigated the effect of these peptides on phagocytes from normal and immunosuppressed mice to elucidate the mechanism of the protective activity of these drugs.

The peptides given to normal mice markedly increased the number of phagocytes in the peripheral blood and peritoneal cavity. Also, FK-156 and FK-565 enhanced the chemotactic, phagocytic and killing activity of peritoneal phagocytes and phagocytic function of the RES. In addition, although the findings are not shown, both peptides enhanced the phagocytic and killing activity of mouse alveolar macrophages and the exudate response of phagocytes to bacteria and phytohemagglutinin inoculated into

the mouse peritoneal cavity. The increase in number of, and the enhancement of functions of phagocytes, by both peptides presumably plays an important role in their strong protective activity.

In addition, these peptides induced interferon, activated complement and increased serum colony stimulating activity (data not shown). However, the involvement of these effects in their protective activity of FK-156 and FK-565 remains to be determined.

Whole bacteria (BCG, *Corynebacterium parvum*), glycolipid (LPS), polysaccharide (glucan, lentinan, krestin) and synthetic compounds (MDP, levamisole, azimexon) have been reported to enhance host resistance to infection[7,15~22]. Their mechanism of action is believed to be to increase the counts and enhance the function of phagocytes. Although whole bacteria and macromolecular compounds may affect phagocytic functions by activating the alternate pathway of complement[5,16~18,23], low molecular-weight compounds have not been reported to activate complement. Lentinan activates peritoneal macrophages only *in vitro*, but does not stimulated the RES[18]. Unlike other low molecular-weight immunostimulants, FK-156 and FK-565 might be characterized by strong *in vivo* and *in vitro* activation of phagocytes and activation of complement.

Both drugs restored impaired host resistance to infection in mice immunosuppressed by cyclophosphamide, hydrocortisone, mitomycin C, carrageenan or tumor[6]. Other investigators have shown that such mice have suppressed host defense mechanisms including humoral and cellular immunity[11,24~26]. The results of the present study also show that these immunosuppressants decreased the counts and functions of phagocytes such as chemotaxis, phagocytosis and killing activity and activity of the RES. Such subversion of phagocyte system is probably important in decreased host resistance to infection. We elucidated that FK-156 restored host resistance to infection by restoration of these subverted factors. Azimexon and krestin were also reported to restore resistance to infection and peripheral leukocyte counts in cyclophosphamide-treated mice[19,22,29]. Our findings suggest that concomitant use of immunoactive peptides with antibiotics may be useful in preventing infection in immunocompromised hosts who do not sufficiently respond to chemotherapy with potent antibiotics.

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