IMMUNOACTIVE PEPTIDES, FK 156 AND FK 565

IV. ACTIVATION OF MOUSE MACROPHAGES

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(Received for publication July 18, 1985)

We investigated the effects of the immunoactive peptides, FK 156 and FK 565, on functions of mouse macrophages. FK 156 and FK 565 given parenterally or orally to mice enhanced spreading of peritoneal macrophages, phagocytosis of latex particles and intracellular killing of bacteria by peritoneal macrophages. FK 156 and FK 565 also enhanced the production of superoxide anion and lysosomal enzyme activities of macrophages. The peptides also activated mouse spleen macrophages, and the kinetics of this activation differed from that of the peritoneal macrophages. In addition, both drugs directly enhanced the production of superoxide anion by mouse peritoneal macrophages treated *in vitro* and enhanced the functions of peritoneal macrophages of athymic nude mice. Both these phenomena suggest that direct activation might be one of the mechanisms of macrophage activation by the peptides.

We reported that FK 156, an immunoactive peptide, isolated from culture filtrates of *Streptomyces olivaceogriseus* sp. nov. and its synthetic analog, FK 565, afforded strong resistance to microbial infections in normal and immunosuppressed mice[1,2], and enhanced the host defense mechanisms against infections[3]. These peptides also had various immunostimulating activities such as adjuvant effects on humoral and cellular immunity and mitogenic activity[4].

Macrophages play an important role not only in host resistance to microorganisms and tumors[5] but also in the regulation of immunity[6]. In this study, we investigated the effects of FK 156 and FK 565 on such functions of macrophages as the spreading on the substratum, phagocytosis, intracellular killing of bacteria, production of superoxide anion and lysosomal enzyme activities.

Materials and Methods

Animals

Four-week old male ICR strain mice and eight-week old male normal and nude Balb/c strain mice were purchased from Japan Clea Co., Ltd.

Immunostimulants

FK 156 and FK 565 were prepared in the Research Laboratories of Fujisawa Pharmaceutical Co., Ltd. (Osaka, Japan). They were dissolved in saline and given to mice in a single intraperitoneal or subcutaneous dose of 1 mg/kg. FK 565 dissolved in 0.5% methylcellulose was given in oral doses of 0.1 mg/kg 6, 5, 4 and 1 day before the experiment.

Preparation of Peritoneal and Spleen Macrophages

Mouse peritoneal cells were collected by the method of BJORNSON[7] without induction. The purity of the resident macrophage preparation was more than 90%. The cells were washed and allowed to adhere to tissue culture dishes in DULBECCO's modified EAGLE's minimum essential medium

(DMEM) containing 10% fetal bovine serum (FBS) for 2 hours. After washing twice with the medium, the adherent cells were assayed for spreading, production of superoxide anion (O_2^-) and lysosomal enzyme activities.

To obtain spleen macrophages, mouse spleens were dispersed to single cells and allowed to adhere to tissue culture dishes for 2 hours. The adherent cells collected with a rubber spatula were washed and assayed for the production of O_2^-.

Phagocytosis

Peritoneal macrophages (1×10^7 cells/ml) were mixed with latex particles (0.08%) in a siliconized test tube and incubated at 37°C for 1 hour with continuous shaking. Then, the macrophages were washed 3 times with saline, smeared on a glass slide, fixed and stained with Giemsa stain. Latex particles engulfed by 100 macrophages were randomly counted in microscopic fields.

Spreading

Peritoneal macrophages at the density of 5×10^4 cells/well in a 24 well-multiwell plate (Nunclon) were adhered by the above method and cultured in fresh medium for 4 more hours. The number of cells with pseudopods per 100 macrophages were counted in microscopic fields.

Intracellular Killing

Intracellular killing activity of macrophages was assayed by the modified method of ZWET[8]. That is, 2×10^7 viable *Pseudomonas aeruginosa* were inoculated into the peritoneal cavity of mice to be engulfed by peritoneal macrophages. After 3 minutes, the peritoneal macrophages were collected and washed to remove free bacteria. The macrophage preparations were incubated at 37°C for appropriate times and then disrupted with saline containing 0.1% sodium dodecyl sulfate (SDS). The released viable bacteria were counted by the agar plating method to determine colony forming units.

Production of O_2^-

Production of O_2^- by macrophages was measured as the reduction of ferricytochrome *c* by the method of JOHNSTON[9]. Phorbol myristate acetate (Sigma Chemical Co.) was used as stimulant at a concentration of 4 μg/ml. For the peritoneal macrophages, the results were expressed as nanomoles of O_2^- released for 90 minutes per mg of adherent cell protein. The protein was determined by the method of LOWRY[10] after lysing the washed adherent macrophages with 0.05% triton X-100. For the spleen macrophages, the experiments were conducted in a suspension culture, and the results were expressed as nanomoles of O_2^- released per 10^7 cells for 90 minutes.

Assay of Lysosomal Enzymes

β-Glucuronidase was assayed by hydrolysis of phenolphthalein-β-glucuronide at pH 4.5[11]. Lysozyme was assayed by the turbidimetric method using *Micrococcus lysodeikticus*[11]. Intracellular enzyme activity was assayed after destruction of cells by 3 freeze-thaw cycles.

Results

Phagocytosis

Peritoneal macrophages from mice intraperitoneally injected with FK 156 engulfed 1.81 times more latex particles than those from the control mice injected with saline (Table 1). Also, peritoneal macrophages from mice orally dosed with FK 565 engulfed 2.15 times more latex particles than the control macrophages.

Intracellular Killing

Relative viable counts of intracellular bacteria after incubation, compared with an initial one, were plotted against incubation time (Fig. 1). Peritoneal macrophages from FK 156- and FK 565-injected mice had significantly greater killing activity on the intracellular bacteria than the control

Table 1. Phagocytosis of latex particles by peritoneal macrophages from FK 156- and FK 565-treated mice.

Drug	No. of ingested particles in 100 macrophages	Stimulation index[a]
FK 156	886±111[b]	1.81
Saline	488±118	
FK 565	909± 78[c]	2.15
5% Methylcellulose	422± 52	

Macrophages were obtained from the peritoneal cavity of mice 4 days after intraperitoneal dosing with 1 mg/kg of FK 156 and 1 day after final oral dosing with 0.1 mg/kg of FK 565. Experiment was done in quadruplicate.

[a] The ratio of the phagocytic activity of macrophages from drug-treated mice to that from control mice.

[b] $P<0.01$.

[c] $P<0.001$.

Table 2. Spreading of peritoneal macrophages from FK 156- and FK 565-treated mice.

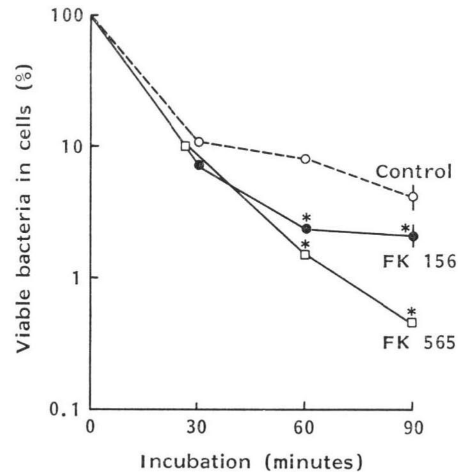
Drug	Spreading (%)
FK 156	89
Saline	22
FK 565	56
5% Methylcellulose	19

Peritoneal macrophages were obtained from mice 4 days after intraperitoneal injection with 1 mg/kg of FK 156 or 1 day after 4 oral dosings with 0.1 mg/kg of FK 565.

Fig. 1. Intracellular killing of *P. aeruginosa* by peritoneal macrophages from FK 156- and FK 565-treated mice.

Peritoneal macrophages were obtained from mice 4 days after intraperitoneal injection of the drugs. The values on the vertical axis express relative viable counts at each incubation time. Experiment was done in duplicate.

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



macrophages at all times tested. In addition, FK 156 enhanced the intracellular killing of *Salmonella enteritidis*, a facultative intracellular bacteria, by peritoneal macrophages (data not shown).

Spreading

Peritoneal macrophages from mice intraperitoneally injected with FK 156 or orally dosed with FK 565 showed markedly enhanced pseudopod formation compared with those from control mice dosed with saline or methylcellulose (Table 2).

Production of O_2^-

Superoxide anion is one of the reactive oxygen intermediates which are produced by phagocytes when responding to such stimuli as phagocytosis of bacteria, and plays a major role in the killing of bacteria[9,12]. Fig. 2 and Table 3 show the production of O_2^- by mouse peritoneal and spleen macrophages. For peritoneal macrophages, FK 156 markedly enhanced the production of O_2^- up to 7 days after intraperitoneal or subcutaneous injection (Fig. 2a). However, for spleen macrophages, FK 156 enhanced the production of O_2^- only 1 day after injection (Fig. 2b). FK 565, given orally, also enhanced the production of O_2^- (Table 3). In addition, FK 156 and FK 565 enhanced the production of O_2^- by peritoneal macrophages treated *in vitro* (Fig. 3).

Lysosomal Enzyme Activity

Among the biochemical aspects of the activities of activated macrophages, the intracellular level

Fig. 2. Production of superoxide anion by peritoneal (a) and spleen macrophages (b) from FK 156-treated mice.

Macrophages were obtained from mice after intraperitoneal or subcutaneous injection of 1 mg/kg of FK 156. Experiment was done in duplicate.

* Significantly different from the values at 0 time ($P<0.05$).

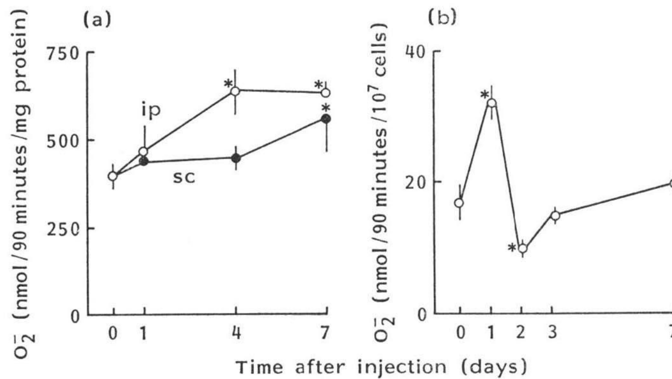


Fig. 3. Production of superoxide anion by mouse peritoneal macrophages treated with FK 156 and FK 565 *in vitro*.

Peritoneal macrophages were obtained from mice 3 days after intraperitoneal injection of thioglycolate medium (1 ml) and treated with the drugs in 10% FBS-DMEM for 20 hours. Experiment was done in duplicate.

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

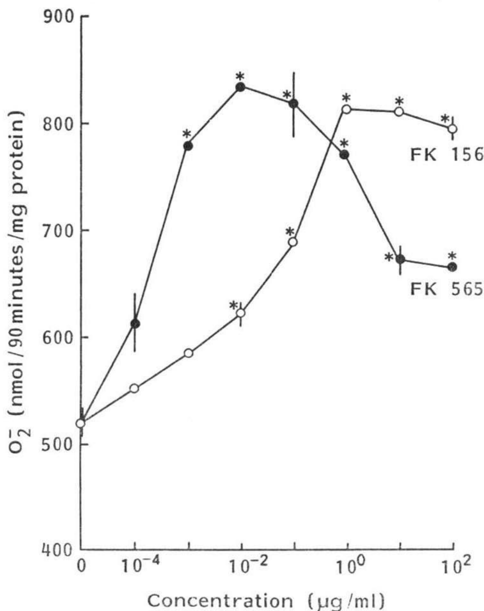


Table 3. Production of superoxide anion by peritoneal macrophages from FK 565-treated mice.

Drug	O_2^- Release (nmol/ 90 minutes/mg)
FK 565	236±19[a]
5% Methylcellulose	130± 4

Peritoneal macrophages were obtained from mice after 4 oral dosings with 0.1 mg/kg of FK 565.

Experiment was done in triplicate.

[a] $P<0.02$.

and extracellular secretion of lysosomal enzymes are known to increase[13,14]. The initial β-glucuronidase level of peritoneal macrophages from the FK 156-treated mice was markedly higher than that from the saline-treated control mice (Fig. 4a). Also, FK 156 enhanced the secretion of the enzyme from macrophages. Similarly, the total and released enzyme levels of lysozyme of macrophages from the FK 156-treated mice were higher than those from the control mice, which indicates that FK 156 enhanced both production and secretion of lysozyme in the peritoneal macrophages (Fig. 4b).

Involvement of T Cells in Macrophage Activation by FK 156

The production of O_2^- and phagocytosis of latex particles by peritoneal macrophages from athymic nude mice injected with FK 156 and FK 565 were studied to determine the involvement

Fig. 4. Lysosomal enzyme activities of peritoneal macrophages from FK 156-treated mice.

Peritoneal macrophages were obtained from mice 4 days after intraperitoneal injection of 1 mg/kg of FK 156. Experiment was done in duplicate. All values for macrophages from FK 156-treated mice were significantly different from the respective values for control macrophages ($P<0.05$).

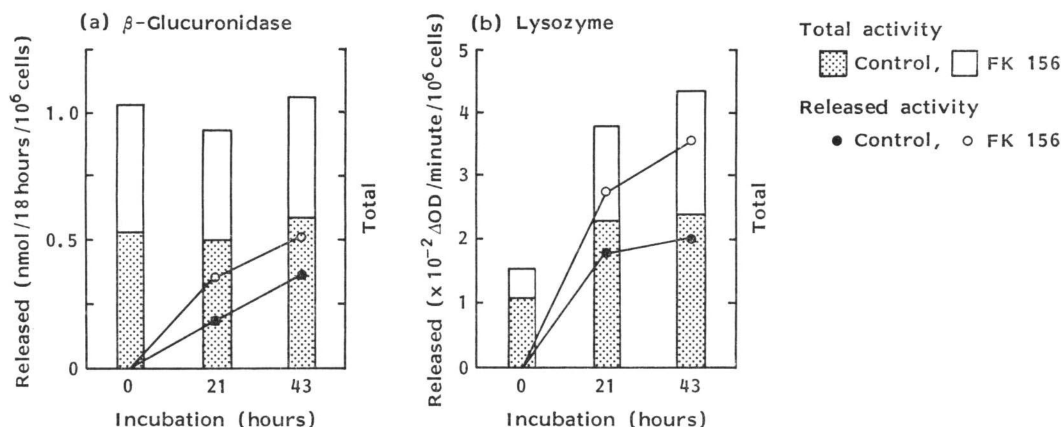


Table 4. Production of superoxide anion and phagocytosis by peritoneal macrophages (Mϕs) from FK 156- and FK 565-treated Balb/c normal and nude mice.

Mouse	Drug	O_2^- Release (nmol/ 90 minutes/mg)	Stimulation index	Phagocytosis (No. of latex/100 Mϕs)	Stimulation index
Nude	FK 156	447±36[b]	1.40	212±35[a]	1.91
	FK 565	488±12[c]	1.53	356±23[c]	3.21
	Saline	319±10		111±19	
Normal	FK 156	354± 4[c]	1.45	284±33	1.93
	FK 565	428±13[c]	1.75	347±17[c]	2.36
	Saline	244±18		147± 1	

Peritoneal macrophages were obtained from mice 4 days after intraperitoneal injection of 1 mg/kg of the drugs. Experiment was done in triplicate.

[a] $P<0.02$.

[b] $P<0.01$.

[c] $P<0.001$.

of T cells in the macrophage activation by the drugs (Table 4). Intraperitoneally injected FK 156 and FK 565 significantly enhanced the production of O_2^- and phagocytosis by peritoneal macrophages from the nude mice as well as from normal mice.

Discussion

In our previous paper, we reported that FK 156 and FK 565, given to normal and immunosuppressed mice, increased the counts of phagocytes in both peripheral blood and peritoneal cavity and enhanced phagocytosis and killing activity of phagocytes[1~3]. In this study, we continued our investigation of the activation of macrophages by these drugs. FK 156 and FK 565 enhanced the phagocytosis of latex particles and intracellular killing of bacteria by mouse peritoneal macrophages. The drugs also enhanced the other functions of macrophages such as spreading on the substratum, production of O_2^-, and increased the intracellular levels and extracellular secretion of lysosomal enzymes. Since it was reported that the reactive oxygen intermediates subsequent to phagocytosis are important to the microbicidal activity of macrophages[15,16], the enhancement of microbicidal activity of macrophages

by FK 156 and FK 565 could be related to the enhanced production of O_2^-. COHN claimed that there were two steps in macrophage activation, a nonspecific inflammatory event and a lymphokine-mediated event, and the macrophages in the latter event expressed microbicidal and tumoricidal activities[17]. Since FK 156 and FK 565 enhanced host resistance to various infections by facultative intracellular and extracellular bacteria[1,2], and their enhancing effects were consistent with the *in vitro* findings, it is suggested that the peptides themselves activated macrophages and this activation was not merely the effect of inflammation.

Fig. 2 shows the different time courses of activation between peritoneal and spleen macrophages by FK 156, and the results for spleen macrophages are consistent with those for the reticuloendothelial system measured by carbon clearance[3]. These findings are of interest because they suggest that the activation of free and fixed macrophages by FK 156 might be induced by different mechanisms.

Although we could not fully elucidate the mechanism of macrophage activation by these peptides, direct activation might be one of the mechanisms of their action judging from the results presented in Fig. 3. However, indirect activation *via* lymphocytes or soluble factors is also a possibility since the activation by FK 156 was relatively slow *in vivo* (see Fig. 2a) compared with muramyldipeptide (MDP) which was reported to enhance the production of O_2^- by peritoneal macrophages a few hours after injection[18,19]. Our findings that FK 156 and FK 565 induced interferon (data not shown) and the observations of GOTOH *et al.* that FK 156 had mitogenic and adjuvant activities[4] support this indirect activation.

It is no doubt that macrophage activation by the immunoactive peptides relates to the enhancement of nonspecific resistance to infection. In addition, this macrophage activation might play a role in other immunological responses such as adjuvant activity, because it is well known that macrophages regulate the immune responses *via* antigen presentation or enhancement of T cell function. Additionally, it is reported that in studies on MDP derivatives, there was a correlation between macrophage activation and adjuvant activity[20]. Therefore, macrophage activation might have an important role in the expression of various biological activities of FK 156 and FK 565.

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