PROTECTIVE EFFECT OF FORPHENICINOL, A LOW MOLECULAR WEIGHT IMMUNOMODIFIER, AGAINST INFECTION WITH *PSEUDOMONAS AERUGINOSA* IN MICE AND ITS MECHANISMS

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(Received for publication June 13, 1986)

The oral administration of forphenicinol increased the survival rate of both normal and immunodepressed mice intraperitoneally or intratracheally infected with clinically isolated strains of *Pseudomonas aeruginosa*. The therapeutic effect of amikacin on intraperitoneal infection with *P. aeruginosa* was enhanced by combined use with forphenicinol.

Forphenicinol did not enhance the bactericidal activity of polymorphonuclear cells (PMN) towards *P. aeruginosa in vitro*, but enhanced it *in vivo*. *In vitro* study indicated that the macrophages taken from mice treated with forphenicinol or the cultured supernatant of these macrophages enhanced the bactericidal activity of PMN. The protective effect of forphenicinol against *P. aeruginosa* infection was thus suggested to be due to macrophage activation followed by the enhancement of the bactericidal activity of PMN.

Forphenicinol, *S*-2-(3-hydroxy-4-hydroxymethylphenyl)glycine, enhances delayed-type hypersensitivity in both normal and immunodepressed mice and phagocytic activity of peritoneal macrophages[1]. Although it had neither tumoricidal nor bactericidal activity *in vitro*, it inhibited the growth of experimental tumors and increased resistance to infection with *Pseudomonas aeruginosa* in mice[2~4].

In this paper, we report on further study of the effect of forphenicinol against *P. aeruginosa* infection and its mechanisms.

Materials and Methods

Animals

Specific pathogen-free female ICR mice were purchased from Charles River Japan, Inc. (Kanagawa) and maintained in a barrier system. They were 5 weeks old at the start of each experiment. In the case of experiments using immunodepressed mice, mice were injected with cyclophosphamide (Shionogi & Co., Ltd., Osaka) or hydrocortisone (Tokyo Kasei Co., Ltd., Tokyo) prior to infection. Cyclophosphamide was given intraperitoneally 4 days before infection at a dose of 50 to 200 mg/kg. Hydrocortisone was given subcutaneously at a dose of 40 mg/kg once daily for 4 days before infection.

Microorganism

Pseudomonas aeruginosa No. 12 which had been clinically isolated by Dr. T. ICHIKAWA, Tokyo Metropolitan Hospital, and kept in Institute of Microbial Chemistry, was employed. In addition, 3 clinical isolates (code; NNα) kindly supplied by Prof. K. MATSUMOTO, Laboratory of Tropical

Medicine, Nagasaki University, Nagasaki) and a clinical isolate (code; CPa) obtained from Tokyo Clinical Research Center, were used in some experiments. Their LD_{50} values in intravenous infection were from 3.0×10^6 to 1.0×10^7 cfu/mouse and were smaller than that of *P. aeruginosa* No. 12 (2.0×10^8 cfu/mouse). Each isolate was grown over night on a tryptic soy agar plate (Eiken, Tokyo), thereafter suspended in saline at the desired concentration. Since *P. aeruginosa* No. 12 tends to lose its virulence after *in vitro* passage of more than 1 month, the bacteria was renewed from lyophilized sources every month. The number of viable *P. aeruginosa* cells (cfu) was counted by spreading 0.1 ml of the cell suspension on nutrient agar plates containing 3% (weight/vol) tryptic soy, 1.7% agar (Difco Laboratories, Detroit, Mich., U.S.A.) and 0.02% cetrimide (Wako Pure Chemicals Ind., Osaka). Cetrimide allowed about 80% of the growth of *P. aeruginosa* at the concentration of 0.02%, however it inhibited the growth of other bacteria.

Forphenicinol and Amikacin

Forphenicinol was synthesized by Banyu Pharmaceutical Co., Ltd., according to the method described by MORISHIMA *et al.*[5] and was given orally at doses of 0.01 to 1.0 mg/kg before or after infection. Amikacin (0.125 or 0.25 mg/mouse, Banyu Pharmaceutical Co., Ltd., Tokyo) was given intramuscularly 1 hour after infection.

Infection

P. aeruginosa suspension of various concentrations (0.1 ml) was injected intravenously or intraperitoneally on day 0. Intratracheal infection was done on day 0 according to the following procedure; the front neck skin was cut and opened surgically with scissors, and the tracheal tract was exposed and injected with 40 μl of the bacterial suspension using a micro-glass syringe. After injection, the skin was retouched with an instant adhesive agent (Aron alpha, Toa Gosei, Tokyo). The protective effect of forphenicinol was expressed in terms of the number of mice surviving 14 days after infection.

Determination of Number of *P. aeruginosa* No. 12 in Mouse Peritoneal Cavity

The cfu of *P. aeruginosa* No. 12 in the peritoneal cavity of mice infected intraperitoneally was determined as follows. The mice were bled to death 1, 3, 6 or 24 hours after infection. The bacteria in the peritoneal cavity of each mouse were collected after intraperitoneal injection of 5.0 ml of sterile saline. Serial 10-fold dilutions of peritoneal fluid were plated and cfu counted.

Preparation of Peritoneal Polymorphonuclear Cells (PMN) and Macrophages

Peritoneal cells were collected from peritoneal fluid of mice 3 hours after intraperitoneal injection of 2 ml of 0.5% glycogen (Nakarai Chemicals, Ltd., Tokyo). These cells were incubated in a plastic dish for 2 hours, thereafter non-adherent cells were collected and used as PMN. Adherent cells were collected from peritoneal fluid of mice injected intraperitoneally with 1.5 ml of 10% Proteose peptone (Difco Laboratories, Mich., U.S.A.) 3 days before sacrifice by excluding non-adherent cells and used as macrophages.

Bactericidal Activity of PMN and Macrophages

PMN or macrophages were suspended with *P. aeruginosa* No. 12 (cell ratio, 4 : 1 to 8 : 1) in EAGLE's minimum essential medium (MEM) containing 5% ICR mouse serum in a CO_2-incubator for 2 hours. The number of remaining viable bacteria was determined as described above.

Determination of Lysozyme and Superoxide Anion in PMN

Lysozyme activity released in the culture medium of PMN was measured according to the method of LITWACK[6]. Egg white lysozyme (Seikagaku-Kogyo, Tokyo) and heat killed *Micrococcus lysodeikticus* (Seikagaku-Kogyo, Tokyo) were used as standard enzyme and substrate. Aliquots of 150 μl of 0.75 mg/ml substrate suspension was incubated with 100 μl of PMN-cultured supernatant at 37°C for 3 hours and the turbidity of the reaction mixture was measured at 540 nm. Superoxide anion production by PMN was measured by the nitroblue tetrazolium (NBT, Sigma, U.S.A.) reduction method described by BAEHNER and NATHAN[7]. The concentration of superoxide dismutase (Sigma, U.S.A.) and phorbol myristate acetate (Sigma, U.S.A.) employed was 0.1 mg/ml and 0.5 μg/ml, respectively.

Statistics

The statistical significances of the survival periods were determined by F-test according to NAKAMURA and KIMURA[8] and Chi-square-test. The other data were analyzed by Student's t-test. A *P*-value of lower than 0.05 was considered to be significant.

Results

Effect of Forphenicinol on *P. aeruginosa* Infection in Normal and Immunodepressed Mice

LD_{50} value of intravenous, intraperitoneal or intratracheal infection with *P. aeruginosa* No. 12 in normal mice was 2.0×10^8, 9.0×10^7 or 2.0×10^7 cfu/mouse, respectively. Mice were the most sensitive to intratracheal infection. Cyclophosphamide reduced host resistance dose-dependently; an especially large reduction was observed in mice infected intratracheally. LD_{50} values in cyclophosphamide (100 mg/kg)-treated mice were 6.0×10^7 (intravenous), 2.3×10^7 (intraperitoneal) and 2.5×10^5 (intratracheal) cfu/mouse. In addition, an LD_{50} value of mice treated with cyclophosphamide (200 mg/kg) was also reduced to 4.0×10^4 cfu/mouse in intratracheal infection. As shown in Table 1, forphenicinol (0.5 mg/kg) increased the survival rate of ICR mice infected with *P. aeruginosa* strain No. 12 intravenously and intratracheally at varied administration schedules. In case of the intratracheal infection, the survival rate of mice given forphenicinol 1 day before the infection went up to 85.7 from 21.4%. Appropriate administration day(s) appeared to be just around the infection. The protective effect of forphenicinol was observed at doses of 0.1 and 0.5 mg/kg when it was given 1 day before intratracheal infection (3.0×10^7 cfu, Table 2). This effective dose range was almost the same as that showing activity in the immune responses and antitumor effects[1~3].

Table 1. Administration schedule of forphenicinol (FPL) and its protective effect on intravenous and intratracheal infection with *Pseudomonas aeruginosa* No. 12.

Administration of FPL (0.5 mg/kg, po)	cfu of *P. aeruginosa* infected[a]	Route of infection[b]	No. of mice survived/ No. of mice treated (%)
Expt 1			
None	3.1×10^8	iv	5/12 (41.7)
Day −3	3.1×10^8	iv	6/12 (50.0)
Day −1	3.1×10^8	iv	9/12 (75.0)[c]
Day −7, −4, −1	3.1×10^8	iv	7/12 (58.3)
Day −5, −3, −1	3.1×10^8	iv	8/12 (66.7)
Expt 2			
None	2.6×10^8	iv	6/12 (50.0)
Day 0	2.6×10^8	iv	10/12 (83.3)[c]
Day 1	2.6×10^8	iv	10/12 (83.3)[c]
Day 0 to 6	2.6×10^8	iv	10/12 (83.3)[c]
Day 1 to 6	2.6×10^8	iv	8/12 (66.7)
Expt 3			
None	3.2×10^7	it	3/14 (21.4)
Day −3	3.2×10^7	it	8/14 (57.1)[d]
Day −1	3.2×10^7	it	12/14 (85.7)[e,f]

[a] *P. aeruginosa* No. 12 was infected on day 0.
[b] iv: Intravenous infection, it: intratracheal infection.
[c] $P<0.05$.
[d] $P<0.01$.
[e] $P<0.001$, by F-test.
[f] $P<0.01$, by X^2-test.

Table 2. Protective effect of forphenicinol (FPL) at varied doses on intratracheal infection with *Pseudomonas aeruginosa* No. 12[a].

FPL (mg/kg)	No. of mice survived/ No. of mice treated (%)
None	0/12 (0.0)
0.01	2/12 (16.7)
0.05	3/12 (25.0)
0.1	6/12 (50.0)[c,d]
0.5	4/12 (33.3)[b]
1.0	1/12 (8.3)

[a] Mice were given FPL orally on day −1 and infected with 3.0×10^7 cfu of *P. aeruginosa* No. 12 intratracheally on day 0.
[b] $P<0.05$.
[c] $P<0.01$, by F-test.
[d] $P<0.05$, by X^2-test.

The protective effect of forphencinol in immunodepressed mice is shown in Table 3. In the case of intratracheal infection with *P. aeruginosa* No. 12 (3.4×10^4 cfu), cyclophosphamide at a dose of 200 mg/kg reduced the survival rate from 100.0 to 0.0%. Forphenicinol given 1 day before infection at an optimal dose (0.5 mg/kg) increased the survival rate up to 36.4%. As for intraperitoneal infection, cyclophosphamide at a dose of 50 mg/kg reduced the survival rate from 100.0 to 16.7% in mice infected with 5.3×10^7 cfu. Hydrocortisone (40 mg/kg) also reduced the survival rate from 44.4 to 16.7% in mice infected with 9.6×10^7 cfu. Forphenicinol (0.5 or 1.0 mg/kg) restored the survival rate in cyclophosphamide-treated or hydrocortisone-treated mice up to 58.3 or 50.0%, respectively. Forphenicinol also increased the survival rate of mice infected with 2 strains (NNα-2844 and CPa-13) out of 4 other clinical isolates with strong virulence (Table 4) in addition to *P. aeruginosa* No. 12.

Table 3. Protective effect of forphenicinol (FPL) on intratracheal or intraperitoneal infection with *Pseudomonas aeruginosa* No. 12 in immunodepressed mice.

FPL (po)		Immuno-depressant (mg/kg)	cfu of *P. aeruginosa* infected[a]	Route of infection[b]	No. of mice survived/ No. of mice treated (%)
mg/kg	Schedule				
Expt 1					
None	—	None	3.4×10^4	it	12/12 (100.0)
None	—	CY 200[c]	3.4×10^4	it	0/12 (0.0)
0.5	Day −1	CY 200	3.4×10^4	it	4/11 (36.4)[g,i]
Expt 2					
None	—	None	5.3×10^7	ip	12/12 (100.0)
None	—	CY 50[c]	5.3×10^7	ip	2/12 (16.7)
0.1	Day −5, −3, −1	CY 50	5.3×10^7	ip	4/12 (33.3)
0.5	Day −5, −3, −1	CY 50	5.3×10^7	ip	7/12 (58.3)[f,h]
1.0	Day −5, −3, −1	CY 50	5.3×10^7	ip	6/12 (50.0)[f]
Expt 3					
None	—	None	9.6×10^7	ip	4/9 (44.4)
None	—	HC 40[d]	9.6×10^7	ip	2/12 (16.7)
0.1	Day −1	HC 40	9.6×10^7	ip	4/12 (33.3)
0.5	Day −1	HC 40	9.6×10^7	ip	6/12 (50.0)[e]
1.0	Day −1	HC 40	9.6×10^7	ip	3/12 (25.0)

[a] *P. aeruginosa* No. 12 was infected on day 0.
[b] it: Intratracheal infection, ip: intraperitoneal infection.
[c] Cyclophosphamide(CY) was injected intraperitoneally on day −4.
[d] Hydrocortisone(HC) was injected subcutaneously on day −4 to −1.
[e] $P<0.05$.
[f] $P<0.01$.
[g] $P<0.001$, by F-test.
[h] $P<0.05$.
[i] $P<0.01$, by X^2-test.

Effect of Forphenicinol in Combination with Amikacin on *P. aeruginosa* Infection

Neither forphenicinol (0.5 mg/kg, day −1) nor amikacin (0.125 mg/mouse) could increase the survival rate of mice ($n=10$) intraperitoneally infected with a large amount of *P. aeruginosa* No. 12 (1.1×10^8 cfu). In this experimental condition, the combination treatment of forphenicinol and amikacin increased the survival rate ($n=10$, $P<0.05$, by F-test) up to 40.0 from 0.0%. Moreover, 60.0% of mice infected with 1.7×10^8 cfu survived ($n=10$, $P<0.01$, by F-test, $P<0.05$, by X^2-test)

Table 4. Protective effect of forphenicinol (FPL) on intravenous infection with clinical isolates of *Pseudomonas aeruginosa*[a].

FPL[b] (mg/kg, po)	No. of mice survived/No. of mice treated (%)			
	Strains of *P. aeruginosa*			
	NNα-2844 2.3×10^6 cfu	CPa-13 3.7×10^6 cfu	NNα-3184 3.5×10^8 cfu	NNα-3091 4.5×10^6 cfu
None	1/12 (8.3)	4/12 (33.3)	3/12 (25.0)	0/12 (0.0)
0.1	3/12 (25.0)	10/12 (83.3)[c,d]	4/12 (33.3)	1/12 (8.3)
1.0	5/12 (41.7)[c]	6/12 (50.0)	5/12 (41.7)	1/12 (8.3)

[a] Each strain of *P. aeruginosa* was infected intraperitoneally at the cfu shown in the heading on day 0.
[b] FPL was given on day 0 to 6.
[c] $P<0.01$, by F-test.
[d] $P<0.05$, by X^2-test.

Fig. 1. Effect of forphenicinol (FPL) on the growth of *Pseudomonas aeruginosa* No. 12 in mouse peritoneal cavity.

Mice ($n=3$) treated with FPL (0.5 mg/kg) orally on day −1 were infected intraperitoneally with *P. aeruginosa* No. 12 (5.0×10^7 cfu) on day 0.

○ cfu of bacteria in the peritoneal cavity of control mice. ● cfu of bacteria in the peritoneal cavity of mice treated with FPL.

* $P<0.05$ against control mice by Student's t-test.

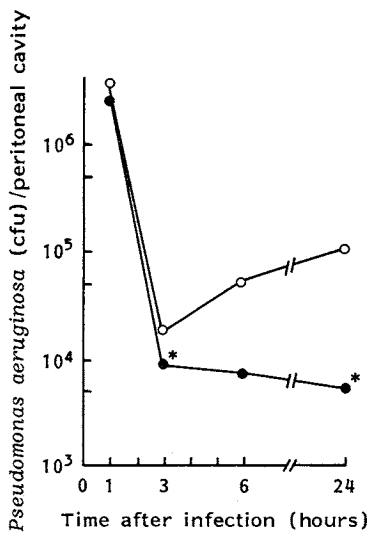
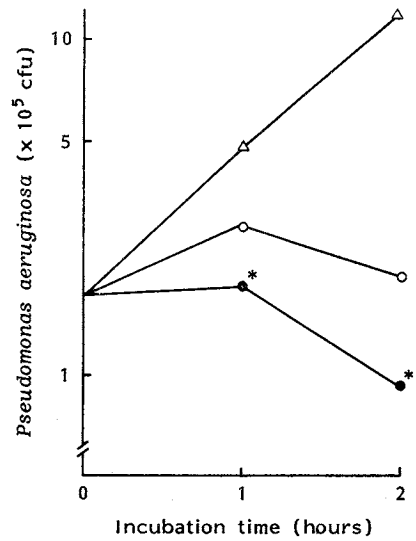


Fig. 2. Killing effect of polymorphonuclear cells (PMN) taken from mice treated with forphenicinol (FPL) on *Pseudomonas aeruginosa* No. 12.

PMN were taken from mice ($n=3$) treated with or without FPL (0.5 mg/kg) 1 day before sacrifice.

△ Without PMN, ○ with PMN taken from control mice, ● with PMN taken from mice treated with FPL.

* $P<0.05$ against PMN taken from control mice by Student's t-test.



when treated with their combination (amikacin, 0.25 mg/mouse). Though, the antibiotic alone was ineffective.

Effect of Forphenicinol on the Growth of *P. aeruginosa* in Peritoneal Cavity

As shown in Fig. 1, oral administration of forphenicinol (0.5 mg/kg) 1 day before intraperitoneal infection with 5.0×10^7 cfu/mouse of *P. aeruginosa* No. 12 decreased the number of bacteria. The number of *P. aeruginosa* No. 12 in the peritoneal cavity of mice given forphenicinol was 0.8×10^4 cfu 3 hours after and 5.0×10^8 cfu 24 hours after infection. It was 47.1 and 5.6% of the control, respectively.

Killing Effect of PMN and Macrophages Taken from Mice Treated with Forphenicinol on *P. aeruginosa*

PMN were incubated with 1.7×10^5 cfu/ml of *P. aeruginosa* No. 12 at a ratio of 8 : 1 for 2 hours at 37°C. Bacteria alone grew up to 1.2×10^8 cfu/ml (Fig. 2). Oral administration of forphenicinol 1 day before sacrifice enhanced the killing activity of PMN and reduced the remaining viable bacteria from 2.0×10^5 cfu/ml (T/C(%), 16.7%) to 9.6×10^4 cfu/ml (T/C(%), 8.0%). On the other hand, macrophages (cell ratio; 8 : 1) from normal mice also reduced the bacterial growth from 1.5×10^5 cfu/ml to 3.8×10^4 cfu/ml (T/C(%), 25.3%, $P<0.05$, by Student's t-test). Oral administration of forphenicinol did not enhance the killing activity of these macrophages.

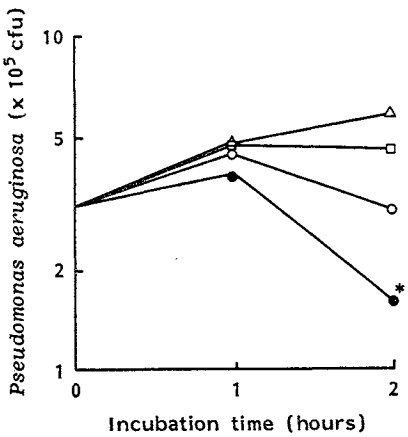
Influence of Forphenicinol on Bactericidal Activity of PMN or Macrophages *in vitro*

PMN or macrophages taken from non-treated mice were cultured with forphenicinol (0.01, 0.1 and 1.0 μg/ml) in MEM containing 10% fetal bovine serum for 1 hour. After washing, the cells were added to *P. aeruginosa* No. 12 suspensions and incubated for 2 hours. Neither the killing activity of PMN nor that of macrophages was enhanced by forphenicinol *in vitro*.

Fig. 3. Killing effect of polymorphonuclear cells (PMN) incubated with peritoneal macrophages (MPh) taken from mice treated with forphenicinol (FPL) on *Pseudomonas aeruginosa* No. 12.

△ Control, □ non-treated PMN, ○ PMN cultured with MPh taken from control mice, ● PMN cultured with MPh taken from mice treated with FPL (0.5 mg/kg) 1 day before sacrifice.

* $P<0.01$ against PMN cultured with MPh taken from control mice by Student's t-test.



Stimulation of Bactericidal Activity of PMN by Macrophages

PMN were cultured with macrophages at a ratio of 1 : 1 in MEM containing 10% fetal bovine serum at 37°C for 24 hours in a CO_2-incubator. After co-culture, the PMN (1.6×10^6 cells) were added to *P. aeruginosa* No. 12 (3.0×10^5 cfu) in 1 ml of MEM containing 5% mouse serum and cultured further for 2 hours. As shown in Fig. 3, *P. aeruginosa* No. 12 grew up to 5.9×10^5 cfu/ml from 3.0×10^5 cfu/ml in the absence of PMN. PMN had a weak bactericidal activity and macrophages taken from normal mice enhanced the bactericidal activity of PMN.

Table 5. Effect of culture fluid of macrophages (MPh) taken from mice treated with forphenicinol (FPL) on the production of lysozyme and superoxide anion by polymorphonuclear cells (PMN).

PMN incubated with culture fluid of	Lysozyme[a]		Superoxide anion	
	μg/ml	T/C (%)	OD_{515}	T/C (%)
None	1.35		0.005	
MPh taken from control mice[b]	1.20	100.0	0.022	100.0
MPh taken from mice treated with FPL[c]	2.00	166.7[d]	0.034	154.5[d]

[a] (Lysozyme activity in PMN-culture-fluid)−(lysozyme activity in MPh-culture-fluid added to PMN culture).
[b] Lysozyme activity of culture fluid of MPh taken from control mice was 1.36 μg/ml.
[c] Lysozyme activity of culture fluid of MPh taken from mice treated with FPL (0.5 mg/kg) 1 day before sacrifice was 2.65 μg/ml.
[d] $P<0.05$, against culture fluid of MPh taken from control mice by Student's t-test.

The number of *P. aeruginosa* No. 12 was reduced from 4.6×10^5 cfu/ml to 3.0×10^5 cfu/ml. The stimulatory effect of macrophages on the bactericidal activity of PMN was more marked in those taken from mice given forphenicinol 1 day before sacrifice (1.6×10^4 cfu/ml, $P<0.01$, PMN cultured with macrophages taken from mice given forphenicinol versus PMNs cultured with macrophages taken from normal mice by Student's t-test). Furthermore 24 hour-culture supernatant of macrophages taken from mice treated with forphenicinol 1 day before sacrifice also enhanced the killing activity of PMN.

Effect of Culture Supernatant of Macrophages on Lysozyme and Superoxide Anion Production of PMN

Macrophages (1.0×10^6 cells/ml) taken from normal or from forphenicinol-treated mice were cultured in serum-free MEM for 24 hours and the cultured supernatant was collected and pooled. PMN (1.0×10^6 cells/ml) taken from normal mice were incubated with the pooled supernatant of macrophage culture for 24 hours and were separated to 5 tubes. The supernatant of macrophage culture taken from forphenicinol-treated mice increased the lysozyme and superoxide anion production by PMN, 66.7 and 54.5%, respectively (Table 5). Moreover, PMN were activated by the cultured supernatant of macrophages incubated with forphenicinol for 24 hours *in vitro*. The superoxide anion of PMN was increased by 36.4% ($P<0.05$, by Student's t-test). Whereas the cultured supernatant of macrophage taken from normal mice did not enhance them. Activity of the supernatant of macrophage culture was not affected by treatment with heating (56°C, 30 minutes) or with acid (pH 2.0).

Discussion

Forphenicinol which has low toxicity[2] is a low molecular weight immunomodifier. We studied the protective effect of forphenicinol against *P. aeruginosa* infection and the mechanisms of action.

P. aeruginosa has been suggested to be a major pathogen in chronic respiratory infections in immunodepressed patients[9,10], who are infected with bacteria easily and repeatedly[11]. The depression in delayed-type hypersensitivity after infection with *P. aeruginosa* has been reported[12,13]. Moreover, it is well known that the efficacy of chemotherapy is reduced in immunodepressed patients. In this case, both macrophages and PMN can play an important role in the host defense mechanisms[14].

ISHIZUKA *et al.*[1] have reported that forphenicinol restores delayed-type hypersensitivity in immunodepressed mice and enhances the activity of peritoneal macrophages. In the present study, we found that the protective effect of forphenicinol against *P. aeruginosa* was associated with the increase in killing activity of PMN through macrophage activation *in vivo*, because PMN were activated by

macrophages taken from mice treated with forphenicinol and by cultured supernatant of macrophages incubated with forphenicinol *in vitro*, but not by forphenicinol directly.

As has been reported previously, forphenicinol enhances the production of γ-interferon and tumor necrosis factor by macrophages[15,16]. As reported in this study we found that forphenicinol stimulates macrophages to produce a heat- and acid-stable factor which activates PMN to inhibit the growth of *P. aeruginosa*. It is known that interleukin 1 is a major product of macrophages and responsible for inflamation. Therefore, we are going to examine whether interleukin 1 enhances PMN or not. While, PENNINGTON *et al.* have reported that human alveolar macrophages produce a PMN-activating factor[17].

Acknowledgment

We thank Prof. K. MATSUMOTO, Laboratory of Tropical Medicine, University of Nagasaki, for his kind gift of clinical isolates of *Pseudomonas aeruginosa*. We also thank Mr. Y. MATSUZAKI and Mr. S. ABE for supplying *Pseudomonas aeruginosa*, Mr. T. MURAI, Mrs. K. ABE and Miss M. NAKADAIRA for their skillful technical assistance.

References

1) ISHIZUKA, M.; S. ISHIZEKI, T. MASUDA, A. MOMOSE, T. AOYAGI, T. TAKEUCHI & H. UMEZAWA: Studies on effects of forphenicinol on immune responses. J. Antibiotics 35: 1042~1048, 1982
2) ISHIZUKA, M.; T. MASUDA, N. KANBAYASHI, Y. WATANABE, M. MATSUZAKI, Y. SAWAZAKI, A. OHKURA, T. TAKEUCHI & H. UMEZAWA: Antitumor effect of forphenicinol, a low molecular weight immunomodifier, on murine transplantable tumors and microbial infections. J. Antibiotics 35: 1049~1054, 1982
3) NITTA, K.; T. TANAKA & M. TAKEUCHI: Effect of forphenicinol, a small molecular immunomodifier, in combination with cyclophosphamide on growth of and immunity to syngeneic murine tumors. Cancer Treat. Rep. 69: 285~291, 1985
4) ISHIBASHI, T.; Y. HARADA, M. TAKAMOTO & A. SHINODA: Immunopotentiation with forphenicinol: Increased resistance to *Pseudomonas* septicaemia in mice. J. Antibiotics 38: 430~432, 1985
5) MORISHIMA, H.; J. YOSHIZAWA, R. USHIJIMA, T. TAKEUCHI & H. UMEZAWA: Synthesis of forphenicinol and forphenicine. J. Antibiotics 35: 1500~1506, 1982
6) LITWACK, G.: Photometric determination of lysozyme activity. Proc. Soc. Exp. Biol. Med. 89: 401~403, 1955
7) BAEHNER, R. L. & D. G. NATHAN: Quantitative nitroblue tetrazolium test in chronic granulomatous disease. N. Engl. J. Med. 278: 971~976, 1968
8) NAKAMURA, E. & M. KIMURA (*Ed.*): Quantal response (all or none type). *In* Bioassay and Biostatistics. pp. 152~158, Hirokawa Publishing Co., Tokyo, 1977
9) KLASTERSKY, J. & D. WEERTS: Recent experience with bacteremia in patients presenting with cancer. Eur. J. Cancer 9: 69~75, 1973
10) DIAZ, F.; L. L. MOSOVITCH & E. NETER: Serogroups of *P. aeruginosa* and the immune response of patients with cystic fibrosis. J. Infect. Dis. 121: 269~274, 1970
11) PENNINGTON, J. E.; H. Y. REYNOLD & P. P. CARBONE: Pseudonomas pneumonia. Am. J. Med. 55: 155~160, 1973
12) COLLIZI, V.; C. GARZELLI, M. CAMPA, L. TOCA & G. FALCONE: Role of specific delayed-type hypersensitivity in *Pseudomonas aeruginosa*-infected mice. Immunology 47: 337~344, 1982
13) PETIT, J. C.; G. RICHARD, B. ALBERT & G. L. DAGUET: Depression by *Pseudomonas aeruginosa* of two T-cell-mediated responses, anti-listeria immunity and delayed-type hypersensitivity to sheep erythrocytes. Infect. Immun. 35: 900~908, 1982
14) MIYAKE, S.; K. NOMOTO, T. YOKOKURA, Y. YOSHIAKI, M. MUTAI & K. NOMOTO: Protective effect of *Lactobacillus casei* on *Pseudomonas aeruginosa* infection in mice. Infect. Immun. 48: 480~485, 1985
15) OKURA, A.; M. NAKADAIRA, K. NAITO, M. ISHIZUKA, T. TAKEUCHI & H. UMEZAWA: Effect of forphenicinol on γ-interferon production in mice sensitized with BCG. J. Antibiotics 39: 569~574, 1986
16) OKURA, A.; M. NAKADAIRA, K. NAITO, H. ARAKAWA, M. ISHIZUKA, T. TAKEUCHI & H. UMEZAWA: Effect of forphenicinol on the production of lipopolysaccharide-induced interferon and tumor necrosis factor, and the growth of Meth A fibrosarcoma in mice. J. Biol. Res. Mod. 6: 28~34, 1987
17) PENNINGTON, J. E.; T. H. ROSSING, L. W. BOETH & T. H. LEE: Isolation and partial characterization of a human alveolar macrophage-derived neutrophil-activating factor. J. Clin. Invest. 75: 1230~1237, 1985