IMMUNOPOTENTIATION WITH FORPHENICINOL: INCREASED RESISTANCE TO *PSEUDOMONAS* SEPTICAEMIA IN MICE

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UMEZAWA *et al.* found that forphenicine, isolated from culture filtrates of *Actinomycetes fulvoviridis* var. *acarbodicus* is a specific inhibitor against chicken intestine alkaline phosphatase and it was shown to enhance delayed type hypersensitivity and antibody formation in mice[1,2]. Thereafter, ISHIZUKA *et al.* found that forphenicinol, a synthetic derivative of forphenicine, enhanced delayed type hypersensitivity and caused macrophage activation by oral administration[3]. Forphenicinol was also shown to exert an antitumor action and to exhibit a protective effect against an experimental *Pseudomonas* infection by mortality study[4]. The present work was designed to study the precise action of forphenicinol against *Pseudomonas* septicaemia in mice.

Female, specific pathogen free ICR mice were obtained from Shizuoka Laboratory Animal Agriculture Cooperative Association, Shizuoka, Japan. Seven- to ten-week old mice were used throughout the experiments.

Forphenicinol was kindly provided by Dr. H. UMEZAWA and Dr. S. OKA, Institute of Microbial Chemistry, Tokyo, Japan. It was prepared by Banyu Pharmaceutical Co., Ltd., Japan. It was dissolved in sterile saline and was administered orally in a volume of 0.1 ml.

Pseudomonas aeruginosa (strain NC-5) was provided originally by Dr. J. Y. HOMMA (Institute of Medical Science, Tokyo University, Japan). This strain was highly virulent for mice and produced neither exotoxin, protease nor lecithinase. Bacterial suspensions were made as described previously[5]. Mice were infected iv with the dose slightly higher than the LD_{50} in a volume of 0.1 ml. Challenged mice were observed for 7 days following infection. Bacterial enumeration in the kidneys was performed as described previously[5]. The viable counts were expressed in $\log_{10}$ units.

Fig. 1. Percent survival of forphenicinol (FP)-treated mice infected with *P. aeruginosa*.

Normal controls and forphenicinol-treated mice which had received the doses indicated of forphenicinol daily for 5 days were infected iv with 5.8×10^7 *P. aeruginosa*.

Each group consisted of 10 mice.

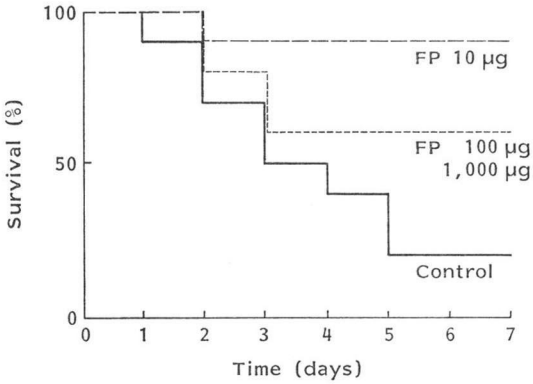
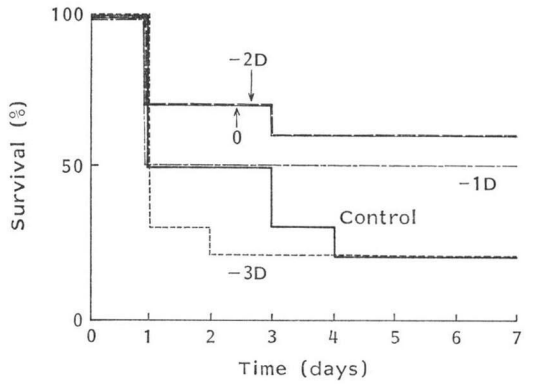


Fig. 2. Percent survival of forphenicinol-treated mice infected with *P. aeruginosa*.

Normal controls and forphenicinol-treated mice which had received 50 μg of forphenicinol at the various times indicated were infected iv with 2.9×10^7 *P. aeruginosa*.

Each group consisted of 10 mice.

D: Day.



Bactericidal assay of peripheral blood was carried out as described previously[6]. Briefly, a mixture of 0.5 ml of heparinized blood and 0.2 ml of the *Pseudomonas* suspension was rotated 150 rpm on a Gyrotory at 37°C for 3 hours. Three hours after incubation, 0.1 ml of the mixture was serially diluted with distilled water and plated on nutrient agar. Bacterial count was carried out after incubation at 37°C for 24 hours.

Nitroblue-tetrazolium (NBT) reduction in casein-induced peritoneal cells was performed

Table 1. The fate of *P. aeruginosa* in the kidneys of forphenicinol treated mice.

Group	No. of mice	Mean of $\log_{10}$ viable *Pseudomonas* in the kidneys
Control	6	3.85±2.34
Forphenicinol-treated	7	2.68±0.78

Normal controls and forphenicinol-treated mice which had received 10 μg of forphenicinol daily for 3 days were infected iv with 4.4×10^7 *P. aeruginosa*.

Bacterial counts were performed in the kidneys of survivors 7 days after infection.

Table 2. Bactericidal activity of peripheral blood of forphenicinol-treated mice against *P. aeruginosa*.

Group	No. of mice	Mean of $\log_{10}$ viable *Pseudomonas*/ 0.1 ml recovered after 3 hours of incubation
Control	5	5.62±0.21
Forphenicinol-treated	5	4.96±0.22*

Normal controls and forphenicinol-treated mice which had received 10 μg of forphenicinol daily for 5 days were sacrificed 1 hour after the last administration of forphenicinol.

Peripheral blood was obtained by heart puncture. Mean number of *Pseudomonas* at zero time was 4.5×10^5 cells/0.1 ml of sample (expressed in $\log_{10}$, 5.65).

* $P<0.005$ in comparison with control.

according to the method of PARK[7]. Peritoneal cells were obtained as described previously[8]. The neutrophils with 10 of more formazan granules were classified as NBT positive cells.

Tests for significance were performed using the Student's t-test. The survival rate was analyzed according to the generalized Wilcoxon test. A P value of less than 0.05 was considered statistically significant.

Firstly, mortality studies were performed in forphenicinol-treated mice which had received the varying doses of forphenicinol daily for 5 days. Each group consisted of 10 mice. All mice were concurrently infected iv with 5.8×10^7 *P. aeruginosa* 1 hour after the last administration of forphenicinol. As can be seen in Fig. 1, forphenicinol-treated mice showed a lower rate of mortality as compared to controls. The difference between the mice receiving 10 μg of forphenicinol for 5 days and controls was significant to $P<0.01$. The next experiment was performed to examine the optimal timing of forphenicinol administration for protection against infection. A single dose of 50 μg forphenicinol was given 1 hour, 1, 2 and 3 days before infection respectively. Mice were infected iv with 2.9×10^7 *P. aeruginosa*. As shown in Fig. 2, the survival rate was higher in mice receiving forphenicinol within 2 days before infection as compared to controls. However, there was no significant difference between control and experimental groups. In the next experiment, the experimental group of mice was given 10 μg of forphenicinol daily for 3 days. Mice were infected iv with 4.4×10^7 *P. aeruginosa* 1 day after the last administration of forphenicinol. Survival was 70% in forphenicinol-treated mice and 60% in controls. The results of bacterial counts in the kidneys of survivors are presented in Table 1. The numbers of bacteria in forphenicinol-treated mice were almost ten times lower than those in controls, although the difference was not statistically significant.

Table 3. Reduction of NBT in casein-induced peritoneal neutrophils of forphenicinol-treated mice.

Group	Mean percentage of NBT positive neutrophils		
	3 hours	7 hours	24 hours
Control	2.4 ±2.2	7.6 ±7.1	15.2 ±10.6
Forphenicinol-treated	27.8 ±16.0*	31.0 ±30.8	35.0 ±16.5

Normal controls and forphenicinol-treated mice which had received 20 μg of forphenicinol daily for 3 days were injected ip with 0.2 ml of 5% sodium caseinate in saline 1 hour after the last administration of forphenicinol.

At the various times indicated after casein injection, peritoneal cells were obtained by washing out the peritoneal cavity with HANK's balanced salt solution containing 5 U/ml of heparin.

Each value represents the mean of five mice.

* $P<0.01$ in comparison with control.

The *in vitro* bactericidal activity of peripheral blood obtained from forphenicinol-treated mice was compared with that of normal controls. The results in Table 2 showed that the number of bacteria in the blood from forphenicinol-treated mice was apparently lower than that of controls.

NBT reduction in casein-induced peritoneal neutrophils was examined. As shown in Table 3,

the numbers of NBT positive cells in forphenicinol-treated mice were clearly higher than those in controls.

The data mentioned above show that pretreatment with forphenicinol increased the resistance against *Pseudomonas* septicaemia in terms of reduction of mortality rate and the number of *in vivo* bacteria. It seems that pretreatment with about 50 μg of forphenicinol per mouse exerted the most protective action against infection. It has been shown by numerous investigators that the resistance to *P. aeruginosa* was based on antibody and phagocytes, mainly polymorphonuclear leucocytes[9~12]. ISHIZUKA *et al.* noted that forphenicinol did not increased antibody production[3]. Our results showed that peripheral blood from forphenicinol-treated mice achieved a more efficient bactericidal effect than that from normal controls. Furthermore, forphenicinol caused a rapid increase in the number of NBT positive neutrophils following casein injection into the peritoneal cavity. It, therefore, seems that the increased resistance of forphenicinol-treated mice against *Pseudomonas* septicaemia would be ascribed to activation of neutrophils. Further studies are needed to clarify whether forphenicinol activates neutrophils directly or through mediation by lymphocytes.

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