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. Thereafter, Ishizuka et al. found that forphenicinol, a synthetic derivative of forphenicine, enhanced delayed type hypersensitivity and caused macrophage activation by oral administration. Forphenicinol was also shown to exert an antitumor action and to exhibit a protective effect against an experimental Pseudomonas infection by mortality study. 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. Mice were infected iv with the dose slightly higher than the LD50 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. The viable counts were expressed in log10 units.

Bactericidal assay of peripheral blood was carried out as described previously. 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.
According to the method of Park7). Peritoneal cells were obtained as described previously8).

The neutrophils with 10 or 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 forphenicol-treated mice which had received the varying doses of forphenicol 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 forphenicol. As can be seen in Fig. 1, forphenicol-treated mice showed a lower rate of mortality as compared to controls. The difference between the mice receiving 10 μg of forphenicol for 5 days and controls was significant to P<0.01. The next experiment was performed to examine the optimal timing of forphenicol administration for protection against infection. A single dose of 50 μg forphenicol 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 forphenicol 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 forphenicol daily for 3 days. Mice were infected iv with 4.4 × 10^7 P. aeruginosa 1 day after the last administration of forphenicol. Survival was 70% in forphenicol-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 forphenicol-treated mice were almost ten times lower than those in controls, although the difference was not statistically significant.

The in vitro bactericidal activity of peripheral blood obtained from forphenicol-treated mice against P. aeruginosa was compared with that of normal controls. Each value represents the mean of five mice.

### Table 1. The fate of P. aeruginosa in the kidneys of forphenicol treated mice.

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean of log_{10} viable Pseudomonas in the kidneys</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.85 ± 2.34</td>
</tr>
<tr>
<td>Forphenicol-treated</td>
<td>2.68 ± 0.78</td>
</tr>
</tbody>
</table>

Normal controls and forphenicol-treated mice which had received 10 μg of forphenicol 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 forphenicol-treated mice against P. aeruginosa.

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean of log_{10} viable Pseudomonas/0.1 ml recovered after 3 hours of incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.62 ± 0.21</td>
</tr>
<tr>
<td>Forphenicol-treated</td>
<td>4.96 ± 0.22*</td>
</tr>
</tbody>
</table>

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

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.

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

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean percentage of NBT positive neutrophils</th>
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<tbody>
<tr>
<td></td>
<td>3 hours</td>
</tr>
<tr>
<td>Control</td>
<td>2.4 ± 2.2</td>
</tr>
<tr>
<td>Forphenicol-treated</td>
<td>27.8 ± 16.0</td>
</tr>
</tbody>
</table>

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

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 μ/ml of heparin.

Each value represents the mean of five mice.

* P<0.01 in comparison with control.
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. Ishizuka et al. noted that forphenicinol did not increase antibody production. 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.

Acknowledgments

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References