STUDIES ON EFFECTS OF FORPHENICINOL ON
IMMUNE RESPONSESMASAAKI ISHIZUKA, SEIJI ISHIZEKI, TORU MASUDA, AYAKO MOMOSE,
TAKAAKI AOYAGI, TOMIO TAKEUCHI and HAMAO UMEZAWAInstitute of Microbial Chemistry
14-23 Kamiosaki 3-chome, Shinagawa-ku, Tokyo 141, Japan

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The oral administration of forphenicinol, *S*-2-(3-hydroxy-4-hydroxymethylphenyl)glycine which was synthesized during the study of derivatives and analogs of forphenicine, augmented delayed-type hypersensitivity to sheep red blood cells and oxazolone in mice. The treatment with forphenicinol restored DTH in mice immuno-suppressed by cyclophosphamide to normal response. Forphenicinol neither augmented antibody-formation nor stimulated proliferation of lymphocytes in the presence or absence of lectins. Phagocytosis by peritoneal macrophages was enhanced by forphenicinol *in vivo* and *in vitro*. Forphenicinol was effective in increasing the production of CFU-C in the presence of colony stimulating factor and partially prevented the reduction of leucocyte counts caused by mitomycin C.

As we reported¹⁾, the screening for inhibitors of enzymes which are located on cell surfaces or membranes resulted in the discovery of low molecular weight immuno-modifiers. In such a study, we found forphenicine, *S*-2-(4-formyl-3-hydroxyphenyl)glycine (Fig. 1)¹⁻³⁾, which inhibited chicken intestine alkaline phosphatase and enhanced delayed-type hypersensitivity (DTH)¹⁾. Forphenicine also increased the number of antibody-forming cells and exhibited inhibition against some murine transplantable tumors¹⁾. These effects *in vivo* were shown by intraperitoneal administration but not by oral administration. Therefore, we synthesized derivatives and analogs and found that forphenicinol, *S*-2-(3-hydroxy-4-hydroxymethylphenyl)glycine, enhanced DTH and exhibited antitumor effect⁴⁾ by oral administration.

In this paper we will report on the action of forphenicinol in augmenting immune responses.

Fig. 1. Structures of forphenicine (R=CHO) and forphenicinol (R=CH₂OH).

Materials and Methods

Mice

CDF₁ mice (Balb/c × DBA/2, female, 8~10 weeks old) were purchased from Shizuoka Laboratory Animal Agriculture Cooperative Association, Shizuoka, Japan. They were kept under filtered fresh air at a constant room temperature of 23 ± 1°C, relative humidity of 55 ± 5%, and fed sterilized mouse pellet (FR-1, Funabashi Farm Co. Ltd., Chiba) and water *ad libitum*.

Forphenicinol and Other Chemicals

Forphenicinol was chemically synthesized. The method of synthesis will be reported on the other paper⁵⁾. It was dissolved in saline or in culture medium. Cyclophosphamide ("Endoxan" Shionogi & Co. Ltd.) and mitomycin C (Kyowa Hakko Co. Ltd.) were dissolved in saline. Concanavalin A (ConA, Pharmacia) and lipopolysaccharide (LPS, Difco Laboratories) were dissolved in culture medium at 0.5 µg/ml and 2 µg/ml respectively.

Immune Responses

Delayed-type hypersensitivity (DTH) to sheep red blood cells (SRBC) or to oxazolone was tested by the method described previously⁶⁾. Forphenicicol was given to mice at the time of immunization.

Antibody formation to SRBC was measured by the hemolytic plaque technic^{7,8)} 4 days after the immunization. Five mice were used in each group.

Blastogenesis of Mouse Spleen Cells

As reported previously for testing the effect of bestatin^{9,10)}, spleen cells were suspended in RPMI 1640 containing 1% fetal calf serum (Lot R. 393423, Gibco, N. Y.) and forphenicicol at the indicated concentrations, and cultured for 3 days in Falcon Microtest plate 3042. When the effect on lectin was tested, ConA or LPS was added at 0.5 $\mu\text{g/ml}$ or at 2 $\mu\text{g/ml}$ respectively. The incorporation of [³H]-thymidine into cultured cells was determined by the method described previously^{9,10)}. Triplicate cultures were done for each datum.

Phagocytosis of Yeasts by Mouse Peritoneal Macrophages

The effect of forphenicicol on phagocytosis by mouse peritoneal macrophages was tested by the method described for the study of bactobolin¹¹⁾. Mice were given forphenicicol orally and 18 hours later, thioglycolate-stimulated peritoneal exudate cells were collected; peritoneal exudate cells were incubated in a plastic dish for 2 hours and adherent cells were prepared; to adherent cells 0.05 ml of yeast suspension (7.5×10^6 cells/ml) was added and incubated for 45 minutes at 37°C in 50% CO₂ in air; after washing thoroughly, adherent cells on the bottom of the plates were stained with May-Grünwald and Giemsa solution and the number of phagocytic cells were counted. Triplicate cultures were done for each datum.

Production of Colony-forming Cells by Bone Marrow Cells *In Vitro*

Bone marrow stem cells were cultured with colony-stimulating factor (CSF) and the colony-forming unit in culture (CFU-C) was counted by the method reported previously¹¹⁾, based on the method described by METCALF¹²⁾, and STANLEY *et al.*¹³⁾ LPS-induced mice serum was used as CSF¹²⁾. Forphenicicol was dissolved in 0.05 ml and added at the start of the culture.

Determination of Leucocyte Counts in Mouse Peripheral Blood

After treatment with mitomycin C (50 $\mu\text{g/mouse}$, daily, i.p.) with or without forphenicicol (10 $\mu\text{g/mouse}$, daily, p.o.) for 5 days, 20 μl of venous blood was taken from the orbital vein by puncture using a sterile glass capillary tube at the day indicated in Fig. 3. The blood was diluted with Isoton containing Zap-Oglobin (Coulter Diagnostics) and leucocytes were counted by a Coulter Counter.

Results

Effect of Forphenicicol on DTH

The effect of forphenicicol on cell-mediated immunity was examined by testing its effect on DTH to SRBC and to oxazolone.

Mice were immunized by subcutaneous injection of 10⁸ SRBC into footpad and were given forphenicicol orally or intraperitoneally. Four days after immunization, DTH response was elicited by subcutaneous injection of 10⁸ SRBC to the footpad of the other side and 24 hours later, the footpad thickness was measured. As shown in Table 1, in both routes of the administration, forphenicicol augmented the DTH response. Compared to the intraperitoneal route, the oral administration was more effective. The enhancement was caused by 1, 10 and 100 $\mu\text{g/mouse}$ orally.

Forphenicicol also augmented oxazolone-induced DTH. As shown in Table 2, the oral administration of from 1 μg to 1 mg/mouse augmented the response.

The effect of forphenicicol in immuno-suppressed mice was examined. Mice were given 6 mg/mouse of cyclophosphamide intravenously once and forphenicicol daily for 3 days. After the treat-

Table 1. Effect of forpheniciniol on the establishment of delayed-type hypersensitivity to sheep red blood cells.*

Immunization	Forpheniciniol ($\mu\text{g}/\text{mouse}$)	I.p. Increase of footpad thickness ($\times 0.1 \text{ mm} \pm \text{S.D.}$)	P.o. Increase of footpad thickness ($\times 0.1 \text{ mm} \pm \text{S.D.}$)	
10^8 SRBC		7.7 ± 2.1	7.9 ± 2.1	
"	1,000	7.0 ± 1.5	10.3 ± 2.9	
"	100	9.3 ± 3.7	12.5 ± 1.6	$P < 0.01$
"	10	10.5 ± 4.5	12.6 ± 2.1	$P < 0.05$
"	1	11.3 ± 2.9	13.7 ± 2.4	$P < 0.05$
"	0.1	13.0 ± 2.5	9.9 ± 1.0	$P < 0.05$

* CDF₁ mice (8 weeks old, female) were immunized with 10^8 SRBC by subcutaneous injection to right footpad of hind paw and administered each dose of forpheniciniol i.p. or p.o. Four days later, mice were challenged with 10^8 SRBC s.c. to left footpad, 24 hours thereafter, the thickness of resulting edema on left footpad was measured by calipers.

Table 2. Effect of forpheniciniol on the establishment of delayed-cutaneous hypersensitivity to oxazolone.*

Immunization	Forpheniciniol ($\mu\text{g}/\text{mouse}$, p.o.)	Increase of footpad thickness ($\times 0.1 \text{ mm} \pm \text{S.D.}$)
Oxazolone	—	3.5 ± 0.11
"	1,000	5.4 ± 0.14 $P < 0.01$
"	100	5.2 ± 0.05 $P < 0.01$
"	10	5.3 ± 0.01 $P < 0.01$
"	1	6.2 ± 0.06 $P < 0.01$
"	0.1	4.7 ± 0.02

* CDF₁ mice (male, 10 weeks old) were immunized by painting 0.1 ml of 5% oxazolone-ethanol solution on shaved abdomen and administered forpheniciniol orally. Two days later, oxazolone was painted on left footpad of hind paw and 48 hours thereafter, the thickness of left hind paw was measured by calipers.

ment, mice were immunized by subcutaneous injection of 10^8 SRBC into the footpad and the DTH response was tested. As shown in Table 3, DTH was strongly suppressed by cyclophosphamide treatment. Daily administration of forpheniciniol 100 $\mu\text{g}/\text{mouse}$ prevented this suppression and restored DTH to an almost normal level. Treatment with 10 $\mu\text{g}/\text{mouse}$ showed partial restoration and no effect was obtained with 1 $\mu\text{g}/\text{mouse}$.

Influence of Forpheniciniol on Antibody Formation to SRBC

The influence of forpheniciniol on antibody formation was tested by the hemolytic plaque technique. Mice were immunized by intravenous

Table 3. Effect of forpheniciniol on the establishment of delayed-type hypersensitivity to sheep red blood cells in mice immuno-suppressed by cyclophosphamide (CY).*

Treatment		Immunization day 0	Increase of footpad thickness ($\times 0.1 \text{ mm} \pm \text{S.D.}$)
day -4	day -3 ~ -1		
CY 6 mg/mouse, i.v.	—	10^8 SRBC	1.2 ± 0.13
"	Forpheniciniol 100 $\mu\text{g}/\text{mouse}$	"	9.4 ± 0.58 $P < 0.01$
"	" 10 "	"	6.5 ± 1.6 $P < 0.05$
"	" 1 "	"	1.1 ± 0.10
None	—	10^8 SRBC	9.5 ± 0.04

* CDF₁ mice (female, 8 weeks old) were injected 6 mg/mouse of cyclophosphamide (CY, Endoxan, Shionogi) intravenously, and 1 day later, they were given 100 μg to 1 $\mu\text{g}/\text{mouse}$ of forpheniciniol orally once a day for 3 consecutive days. One day after the last administration, mice were immunized with 10^8 SRBC and DTH response was determined as described in Table 1.

Fig. 2. Effect of forphenicidin on phagocytosis of yeasts by peritoneal macrophages.

Forphenicidin was given p.o. to CDF₁ mice 24 hours before assay.

to 1 mg/mouse, the maximum stimulation was shown by 100 μg/mouse.

injection of 10⁸ SRBC and given forphenicidin at the time of the immunization. Four days later, antibody formation was determined by enumerating the direct PFC in spleens. Forphenicidin from 1 μg to 1 mg/mouse intraperitoneally or orally did not produce any effect on the number of antibody-forming cells.

Effect of Forphenicidin on Phagocytosis of Yeasts by Mouse Peritoneal Macrophages

Mice were injected with thioglycolate broth intraperitoneally, and 3 days later, they were given forphenicidin orally. One day thereafter, macrophage cultures were prepared from peritoneal exudate cells and incubated with yeasts. After the incubation, the number of phagocytic cells was counted by a microscope. Fig. 2 shows that phagocytic activity of macrophages was stimulated by administration of forphenicidin. Although the effect was observed in doses of 1 μg

Effect of Forphenicidin on Lymphocyte Proliferation with or without Mitogens

The influence of forphenicidin on proliferation of mouse lymphocytes was examined. Forphenicidin was added at from 0.025 μg to 100 μg/ml to spleen cell cultures with or without ConA or LPS and incubated for 3 days. After the incubation, incorporation of [³H]thymidine into cultured cells was determined. No change in the incorporation was observed due to the addition of forphenicidin to the cultures (Table 4).

Spleen cells were incubated with forphenicidin for 24 hours. After incubation, lymphocytes were collected and cultured for 3 days, and the incorporation of [³H]thymidine into cells was determined⁹⁾. In this system also, forphenicidin produced no effect on lymphocyte proliferation.

Mice were given forphenicidin (1 μg~1 mg/mouse) orally. One day later, spleen cells were col-

Table 4. Influence of forphenicidin on blastogenesis of spleen cells.

Forphenicidin (μg/ml)	Without lectin	ConA (cpm/culture ± S.D.)	LPS
None	147 ± 35	19,305 ± 312	11,082 ± 963
0.025	167 ± 49	19,975 ± 1,908	10,773 ± 650
0.1	107 ± 11	18,317 ± 1,686	10,857 ± 1,034
0.4	158 ± 28	20,974 ± 1,495	10,600 ± 840
1.6	124 ± 14	20,237 ± 1,468	12,177 ± 920
6.2	133 ± 15	20,645 ± 1,145	12,121 ± 947
25	130 ± 14	21,727 ± 2,322	11,458 ± 1,304
100	143 ± 10	19,786 ± 1,370	11,240 ± 793

Table 5. Effect of forphenicidinol on the production of CFU-C (granulocytes-macrophages) from mouse bone marrow cells stimulated by LPS-induced colony-stimulating factor (CSF).*

Forphenicidinol ($\mu\text{g/ml}$)	1% CSF No. of CFU-C	2% CSF No. of CFU-C
10	54.3 ± 3.9 $P < 0.05$	—
1	68.0 ± 1.6 $P < 0.01$	125.0 ± 6.4 $P < 0.05$
0.1	47.3 ± 6.8 $P < 0.05$	123.5 ± 2.1 $P < 0.05$
0.01	44.0 ± 5.7	116.0 ± 12.2 $P < 0.05$
0.001	43.0 ± 3.3	96.3 ± 13.1
0	37.0 ± 0.8	79.7 ± 12.1

* Mouse bone marrow cells were suspended in alpha-MEM supplemented with those as described by STANLEY *et al.*¹³⁾ and cultured with forphenicidinol in the presence of CSF for 10 days at 37°C in 10% CO₂. The number of CFU-C was counted.

lected and cultured for 3 days. After 3 days, the incorporation of [³H]thymidine was tested. Forphenicidinol produced no effect.

Augmentation of CFU-C Production in Mouse Bone Marrow Cell Culture by Forphenicidinol

In order to know whether forphenicidinol affects bone marrow progenitor cells, we examined the effect of forphenicidinol on CFU-C in bone marrow cell cultures. The colonies which are produced in the presence of LPS-induced CSF were known to be granulocytes and macrophages. Table 5 shows that forphenicidinol at 1 $\mu\text{g/ml}$ increased the number of CFU-C by 84% in 1% CSF-medium and by 57% in 2% CSF-medium. The effect of forphenicidinol was observed also at 0.1 or 0.01 $\mu\text{g/ml}$. Moreover, 10 $\mu\text{g/ml}$ caused no damage of bone marrow cells.

Prevention of Mitomycin C-Induced Leucopenia in Mice by Forphenicidinol

To determine the effect of forphenicidinol on leucocytes count in mice to which mitomycin C was intraperitoneally injected was examined. Mitomycin C (50 $\mu\text{g}/\text{mouse}$, i. p.) was given to mice daily for 5 days with or without forphenicidinol and the number of leucocytes in peripheral blood was counted from 1 day after the last administration. The result is shown in Fig. 3. The leucocyte count of mice treated with mitomycin C alone was reduced to about 6,000 mm^3 1 or 2 days after the last injection and was further reduced up to the time of death. In the group treated with mitomycin C and forphenicidinol (10 $\mu\text{g}/\text{mouse}$ daily during mitomycin C treatment) the leucocyte

Fig. 3. Effect of forphenicidinol on mitomycin C induced leucopenia in mice.

count was also reduced to about 5,000 mm³ 1 day after the last administration, but no further decrease was observed up to 7 days. The mitomycin C-treated mice started to die from 4 days and within 9 to 12 days, all mice had died. In comparison, the survival period of mice was prolonged by forphenic-inol treatment (Fig. 2).

Discussion

Forphenicine inhibited chicken intestine alkaline phosphatase strongly and other alkaline phosphatases weakly¹⁾. The kinetic data were interesting and indicated an uncompetitive inhibition with the substrate. Therefore, we tested the immuno-modifying effect of all derivatives even those which had no activity in inhibiting alkaline phosphatase. Forphenic-inol which did not inhibit the enzymes was found to enhance immune responses in mice. A preliminary test using [³H]-labeled forphenic-inol suggested binding to mouse lymphocytes and macrophages.

The establishment of DTH to SRBC and oxazolone was augmented by forphenic-inol, especially by oral administration in a wide dose range. Optimum enhancement was observed at from 1 to 100 µg/mouse. In mice immuno-suppressed by cyclophosphamide, higher doses such as 10 and 100 µg/mouse were required to restore the reduced DTH. Forphenic-inol from 1 µg to 1 mg/mouse did not produce any effect on antibody formation. Thus, forphenic-inol was shown to be an immuno-modifier of cell-mediated immunity.

Forphenic-inol was found to have no effect on lymphocyte proliferation. As reported previously, bestatin¹⁰⁾ and diketocoriolin B¹⁴⁾ has stimulatory effect on lymphocyte proliferation, and other low molecular weight immuno-modifiers are also known to cause blastogenesis of lymphocytes^{15,16)}.

As shown in Fig. 1, being different from bestatin, forphenic-inol activated macrophages *in vivo* and *in vitro*. This effect suggests that the primary site of augmentation of immune response by forphenic-inol might be macrophages. In preliminary experiments, forphenic-inol stimulated the production of superoxide anion by macrophages and changed the activities of some enzymes in macrophages.

Besides the enhancement of DTH and macrophage phagocytosis, forphenic-inol increased the production of CFU-C in bone marrow cell cultures with CSF. This effect was reflected in the prevention of reduction of the number of leucocytes in animals with leucopenia. As shown in Fig. 2, the treatment with forphenic-inol counteracted partially the decrease of leucocyte counts caused by mitomycin C treatment.

Forphenicine enhanced DTH and increased the number of antibody-forming cells in a wide range of doses given by intraperitoneal administration²⁾. Forphenicine also increased CFU-C. Moreover, forphenicine was shown to act on macrophages and increase the number of antibody-forming cells: when lymphocytes or macrophages were treated with forphenicine and cultured with untreated macrophages or lymphocytes, the effect in increasing antibody-forming cells was observed in the reconstituted culture of lymphocytes with macrophage incubated with SRBC and forphenicine. Thus, the minor structural change from forphenicine to forphenic-inol eliminated the effect of increasing the number of antibody-forming cells and causing the blastogenesis of peripheral lymphocytes. These observations suggest that further derivative and analog studies around forphenicine should be done.

Forphenicine and forphenic-inol have extremely low toxicity. The clinical study has indicated that forphenic-inol orally given increases T cell % and enhances NK cell activity in cancer patients¹⁷⁾.

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