EFFECT OF FORPHENICINOL ON γ-INTERFERON PRODUCTION IN MICE SENSITIZED WITH BCG

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Forphenicinol stimulated the production of BCG-induced γ-interferon in BCG-sensitized mice as much as 11-fold whereas it did not induce interferon production in unsensitized mice. This stimulatory effect was also observed in mice immuno-suppressed by cyclophosphamide. Furthermore, BCG-induced γ-interferon production was reduced in mice by treatment with an anti-macrophage agent (silica gel), and forphenicinol administration into such mice could augment their interferon production. Transplantation of macrophages from forphenicinol-treated mice into mice injected with silica gel remarkably enhanced the BCG-induced interferon production in the recipients. These results suggest that the stimulatory effect of forphenicinol on BCG-induced γ-interferon production in mice was due to the activation of macrophages.

As reported previously[1~3], forphenicinol, *S*-2-(3-hydroxy-4-hydroxymethylphenyl)glycine, augments delayed-type hypersensitivity to sheep red blood cells and to oxazolone, and exhibits antitumor activity against murine transplantable tumors through activation of host defense mechanisms. Recently, studies have accumulated to show that most immuno-modifiers stimulate the production of cytokines, including IL-1, IL-2 and interferons, which modulate the immune system[4]. In this paper, we report on the effect of forphenicinol on interferon production in mice.

Materials and Methods

Mice

Specific pathogen-free female ICR mice were purchased from Charles River Japan, Inc. (Kanagawa) and were maintained in a barrier system. They were 6 weeks old at the start of each experiment.

Cell and Virus

Mouse LY cells were used for interferon assay and were maintained in Eagle's minimum essential medium supplemented with 5% calf serum. They were passaged every 3 days. The New Jersey strain of vesicular stomatitis virus (VSV) was prepared by infecting BHK 21 cells at a low multiplicity. The culture fluid was harvested after overnight incubation and stored at −80°C.

Forphenicinol and Other Reagents

Forphenicinol was chemically synthesized by Banyu Pharmaceutical Co., Ltd. according to the methods described by MORISHIMA *et al.*[5]. Lyophilized vaccine of viable *Mycobacterium bovis* strain BCG was purchased from Japan BCG Manufacturing Co. (Tokyo). Silica gel (Art. 7731) was obtained from E. Merck (Darmstadt) and finely ground (<5 μm). Cyclophosphamide (Endoxan) and [5,6-^{3}H]-

uridine were purchased from Shionogi & Co., Ltd. (Osaka) and Amersham (England), respectively.

Preparation of Macrophages

Macrophages were prepared from spleens of ICR mice. A single splenic cell suspension was made, and the cells were allowed to adhere onto plastic dishes for 1 hour. Adherent cells were harvested using a rubber policeman and used as splenic macrophages.

Induction of Interferon by BCG in Mice

The BCG vaccine was suspended in saline at 5 mg/ml and 0.2 ml aliquots of the suspension (containing about 10^7 viable cells) were intravenously injected into mice. Two weeks later, the mice received an intravenous injection of BCG at a dose of 10^7 or 10^6 cells/mouse and were aseptically bled by cardiac puncture 2 or 3 hours thereafter[6,7]. The sera obtained from groups of 3 or 6 mice were stored at −80°C until assayed.

Interferon Assay

Interferon activity in mouse sera was assayed according to slight modifications of the methods based on the inhibition of viral RNA synthesis[8] or the inhibition of cytopathic effect[9], using LY cells and VSV. For the former, LY cells plated into wells of 96-well microtest plates at 10^5 cells/well were incubated with medium containing serial dilutions of interferon samples. After overnight incubation at 37°C, the cells were infected with VSV (input multiplicity of 10 PFU/cell) in the presence of 2.5 μg/ml of actinomycin D, and 1 hour later, 50 mμCi of [^{3}H]uridine was added to each well. The cells were incubated for another 4 hours. Radioactivity incorporated into the cells was measured by usual methods. Inhibition of cytopathic effect was measured as follows: LY cells treated with interferon samples as described above were infected with VSV at a multiplicity of 1 PFU/cell. After 24-hour incubation at 37°C, the cells were stained with 0.02% crystal violet. After extraction of the fixed dye with alcohol, the absorbance of the extract from each well was measured at 590 nm with a spectrophotometer (ImmunoReader NJ-2000, Nippon InterMed K.K.). A reference standard of mouse interferon (type I) was included in every assay and all titers were expressed as international units per ml in terms of a National Institute of Health standard preparation.

Results

Interferon-inducing Activity of Forphenicinol

ICR mice were injected with forphenicinol at doses from 0.5 to 50 mg/kg intraperitoneally or administered the drug orally at a dose of 0.5, 1.0 or 10 mg/kg. They were then bled by cardiac puncture 2, 4, 8, 16, or 24 hours later. Interferon activity found in all the samples was less than 10 IU/ml.

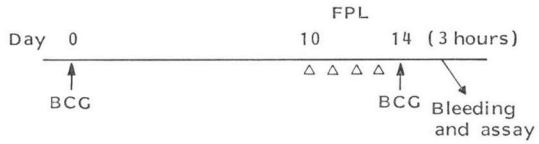
Effect of the Drug on the Production of γ-Interferon Induced by BCG in BCG-sensitized Mice

ICR mice ($n=3$) were immunized by intravenous injection of 10^7 or 10^6 BCG cells on day 0 and were given 0.01, 0.1, or 1.0 mg/kg of forphenicinol intraperitoneally once daily for 4 days from day 10 to 13. On day 14, they were injected with 10^7 or 10^6 BCG cells and then bled 2 hours thereafter. Interferon activity was determined in terms of the pooled sera taken from mice of each group. As shown in Table 1, 6,630 IU/ml of interferon was detected in the sera of mice immunized and elicited with 10^7 BCG cells. The administration of 0.1 mg/kg of forphenicinol almost doubled the interferon activity.

When elicited with 10^6 BCG cells, interferon production was only 50 IU/ml. In this case, forphenicinol in doses of 0.01 and 0.1 mg/kg stimulated the production 11- and 7-fold, respectively. However, when mice were sensitized with 10^6 BCG cells, they did not produce interferon.

The interferon contained in the sera of mice treated with a large dose of BCG and forphenicinol was inactivated 91% by dialysis overnight against pH 2 buffer, but remained 95% active after being heated at 56°C for 30 minutes. These properties were the same as those of the interferon in sera of

Table 1. Effect of forphenicinol (FPL) on the production of BCG-induced interferon in mice.



Sensitizing BCG, iv	Eliciting BCG, iv	FPL mg/kg, ip	Interferon in serum (IU/ml)
10^7	10^7	0	6,630
〃	〃	0.01	3,080
〃	〃	0.1	11,750
〃	〃	1.0	7,020
〃	10^6	0	50
〃	〃	0.01	542
〃	〃	0.1	373
〃	〃	1.0	<30
10^6	10^7	0	<30
〃	〃	0.01	<30
〃	〃	0.1	<30
〃	〃	1.0	<30

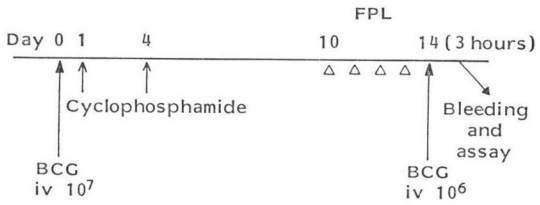
Mice: ICR female, 6 weeks old, $n=3$.
Interferon assay: Inhibition of RNA synthesis of VSV in LY cells.

mice treated with BCG (10^7) alone, and they were thus shown to be γ-interferon[8,10].

Effect of the Drug on BCG-induced Interferon Production in Cyclophosphamide-treated Mice

ICR mice ($n=3$) were immunized by the intravenous injection of 10^7 BCG cells on day 0, and 14 days thereafter, interferon was elicited by 10^6 BCG cells. Sera obtained from each group of mice were pooled before assay. As shown in Table 2, though the interferon activity in normal mouse sera was estimated to be 50 IU/ml, that in the sera obtained from mice given intraperitoneal injections of 100 mg/kg of cyclophosphamide twice (days 1 and 4) was less than 30 IU/ml. Whereas mice administered 100 mg/kg of cyclophosphamide and 0.1 mg/kg of forphenicinol produced 139 IU/ml of interferon.

Table 2. Effect of FPL on the production of BCG-induced interferon in immuno-suppressed mice.



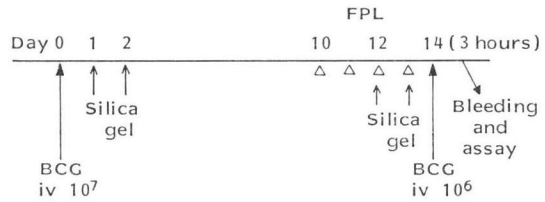
Cyclophosphamide mg/kg, ip	FPL mg/kg, ip	Interferon in serum (IU/ml)
0	0	50
100	0	<30
〃	0.01	64
〃	0.1	139
〃	1.0	<30

Mice: ICR female, 6 weeks old, $n=3$.
Interferon assay: Inhibition of RNA synthesis of VSV in LY cells.

Effect on BCG-induced Interferon Production in Silica-treated Mice

ICR mice ($n=6$) sensitized with 10^7 BCG cells on day 0 were injected intravenously with 1 mg/kg of silica gel on days 1, 2 and/or days 12, 13. They were treated by oral administration of forphenicinol for 4 days (day 10 to 13) at a dose of 0.5 mg/kg, and interferon was elicited by the injection of 10^6 BCG cells on day 14. Three hours later they were bled and their serum interferon activity was measured indi-

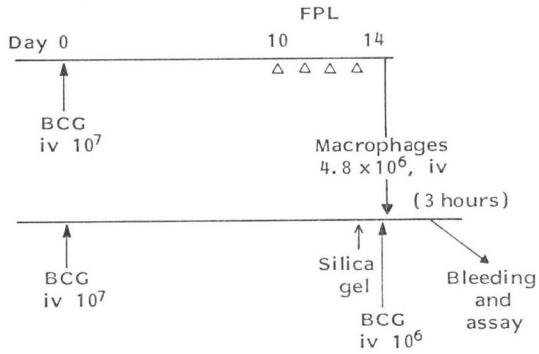
Table 3. Effect of FPL on BCG-induced interferon production in mice injected with silica gel.



Treatment			Interferon in serum	
BCG, iv day 0, 14	Silica gel 1 mg, iv	FPL 0.5 mg/kg, po	IU/ml	T/C (%)
—	—	—	0	
+	—	—	302	
+	Day 1, 2	—	265	100
+	〃	+	942	355
+	Day 12, 13	—	30	100
+	〃	+	130*	433
+	Day 1, 2, 12, 13	—	57	100
+	〃	+	300**	526

*$P<0.01$, **$P<0.05$ by U-test.
Mice: ICR female, 6 weeks old, $n=6$.
Interferon assay: Inhibition of cytopathic effect of VSV in LY cells.

Table 4. Effect of macrophages treated with FPL on BCG-induced interferon production.



BCG, iv	Silica gel 1 mg, iv	Macrophage transfer, iv	Interferon in serum	
			IU/ml	T/C (%)
—	—	—	0	
+	—	—	302	
+	+	—	8	100
+	+	+ (Control)	53	663
+	+	+ (FPL-treated)	115	1,438

Mice: ICR female, 6 weeks old, $n=6$.
Interferon assay: Inhibition of cytopathic effect of VSV in LY cells.

vidually. As Table 3 shows, injection of the anti-macrophage agent reduced the BCG-induced interferon production, which was the least when the mice were injected with the silica gel on days 12 and 13. Forphenicinol significantly ($P<0.05$ by U-test) stimulated interferon production in the mice injected

with silica gel, from 3.6- to as much as 5.3-fold over the control level.

Effect of Transfer of Macrophages from Forphenicinol-treated Mice on BCG-induced Interferon Production in Mice Injected with Silica Gel

ICR mice ($n=6$) were injected with 10^7 BCG cells and 1 mg/kg of silica gel on day 0 and day 13, respectively. On day 14, they were injected intravenously with 10^6 BCG cells and 4.8×10^6 splenic macrophages at the same time; these macrophages had been taken from another ICR mice ($n=12$) sensitized with 10^7 BCG cells on day 0 and given 0.5 mg/kg of forphenicinol orally for 4 days from day 10 to 13. To determine the individual interferon activity, the mice receiving the macrophages were bled 3 hours thereafter. As shown in Table 4, the interferon titer in sera from mice which did not receive foreign macrophages was only 8 IU/ml, while sera from mice transferred with macrophages from untreated mice contained 53 IU/ml of interferon. Forphenicinol treatment of the donor cells stimulated the interferon production in the recipients up to a level of 115 IU/ml.

Discussion

The influence of forphenicinol on interferon production was investigated. Forphenicinol alone could not induce interferon in normal mice at doses which were effective in enhancing immune responses or in treating murine tumors[1~3]. Much work indicates that macrophages have an important role in interferon production[10~13]. As reported previously[1], forphenicinol is thought to enhance immune responses through macrophage activation. It is known that mice sensitized and elicited with 10^7 BCG cells at a 2-week interval produce a high level of γ-interferon[6]. Using this schedule, we have shown that the administration of forphenicinol at doses of 0.1 to 1.0 mg/kg markedly increases interferon production. Although the triggering by 10^6 BCG cells induced only a small amount (50 IU/ml) of interferon, forphenicinol treatment under these conditions stimulated the production more than 10-fold. In mice sensitized with 10^6 BCG cells, interferon was not induced by elicitation with 10^7 BCG cells, and interferon production was not enhanced by forphenicinol administration for 4 days before the eliciting dose was given. Therefore, the enhancing effect of forphenicinol can only be seen in mice primed with 10^7 BCG cells.

The effect of forphenicinol was moderate in mice challenged with 10^7 BCG cells because elicitation with 10^7 BCG cells gives the optimal response[6]. Forphenicinol partly substituted for the immunological functions of BCG cells injected to elicit interferon production in the case when the elicitation was carried out with 10^6 cells. Forphenicinol obviously restored the interferon production in mice whose immunity was suppressed by cyclophosphamide injection.

In reviewing these results, it is obvious that forphenicinol can stimulate BCG-induced γ-interferon production in mice by augmenting immune responses systemically. Therefore, we examined whether or not the stimulatory effect of forphenicinol was due to its macrophage activating capacity. The experimental results revealed that macrophages do indeed play an important role in BCG-induced γ-interferon production in mice and suggest that the effect of forphenicinol on γ-interferon production is mediated through macrophage activation.

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) HERSH, E. M.; M. A. CHIRIGOS & M. J. MASTRANGELO (*Ed.*): Progress in Cancer Research and Therapy. Vol. 16, Augmenting Agents in Cancer Therapy. Raven Press, New York, 1981
5) MORISHIMA, H.; J. YOSHIZAWA, R. USHIJIMA, T. TAKEUCHI & H. UMEZAWA: Synthesis of forphenicinol and forphenicine. J. Antibiotics 35: 1500~1506, 1982
6) SALVIN, S. B.; J. S. YOUNGNER & W. H. LEDERER: Migration inhibitory factor and interferon in the circulation of mice with delayed hypersensitivity. Infec. Immun. 7: 68~75, 1973
7) YOUNGNER, J. S. & W. R. STINEBRING: Interferon appearance stimulated by endotoxin, bacteria, or viruses in mice pre-treated with *Escherichia coli* endotoxin or infected with *Mycobacterium tuberculosis*. Nature 208: 456~458, 1965
8) SUZUKI, J.; T. AKABOSHI & S. KOBAYASHI: A rapid and simple method for assaying interferon. Jpn. J. Microbiol. 18: 449~456, 1974
9) STEWART, W. E., II: Interferon assays. *In* The Interferon System. *Ed.*, W. E. STEWART, II, pp. 13~26, Springer-Verlag, Wien, New York, 1979
10) NEUMANN, C.; E. MACHER & C. SORG: Interferon production by *Corynebacterium parvum* and BCG-activated murine spleen macrophages. Immunobiology 157: 12~23, 1980
11) EPSTEIN, L. B.; M. J. CLINE & T. C. MERIGAN: The interaction of human macrophages and lymphocytes in the phytohemagglutinin-stimulated production of interferon. J. Clin. Invest. 50: 744~753, 1971
12) MILSTONE, L. M. & B. H. WAKSMAN: Release of virus inhibitor from tuberculin-sensitized peritoneal cells stimulated by antigen. J. Immunol. 105: 1068~1071, 1970
13) NEUMANN, C. & C. SORG: Immune interferon. 1. Production by lymphokine-activated murine macrophages. Eur. J. Immunol. 7: 719~725, 1977