EFFECT OF FORPHENICINOL ON THE PRODUCTION OF Ia-POSITIVE MACROPHAGES IN MICE WITH OR WITHOUT L1210 LEUKEMIA AND ON THE GROWTH OF L1210 IN IMMUNIZED MICE

Akira Okura*, Hiroharu Arakawa, Tomoko Yoshinari and Kyozo Naito

Central Research Laboratories, Banyu Pharmaceutical Co., Ltd.,
2-9-3 Shimomeguro, Meguro-ku, Tokyo 153, Japan

Masaaki Ishizuka, Tomio Takeuchi† and Hamao Umezawa†

Microbial Chemistry Research Foundation,
Institute for Chemotherapy,
18-24 Motono, Miyamoto, Numazu-city, Shizuoka 410-03, Japan
†Institute of Microbial Chemistry,
3-14-23 Kamiosaki, Shinagawa-ku, Tokyo 141, Japan

(Received for publication December 25, 1985)

Oral administration of forphenicinol, *S*-2-(3-hydroxy-4-hydroxymethylphenyl)glycine, increased the production of Ia-positive peritoneal macrophages in healthy mice. Moreover, forphenicinol increased Ia-positive macrophages in L1210-bearing mice. Though forphenicinol was ineffective against mouse leukemia L1210 when administered alone, in combination with L1210 vaccine it prolonged the survival time of L1210-bearing mice and the increase in the number of Ia-positive macrophages in peritoneal cavity coincided with the prolongation of the survival time. These results suggest that the initial action of forphenicinol is the preferential induction of Ia-positive macrophages, which play a role in the subsequent activation of various immune responses including macrophage activation.

Forphenicinol is a low molecular weight immunomodifier which is effective in inhibiting tumor growth[1,2] and bacterial infections in mice[1,3]. It stimulates phagocytosis of macrophages and augments delayed-type hypersensitivity to sheep red blood cells and to oxazolone[4]. The efficacy of forphenicinol on tumors and bacteria had been suggested to be due to macrophage activation, although the details of this mechanism have not yet been worked out.

In this paper we report the effect of forphenicinol on induction of Ia-positive macrophages.

Materials and Methods

Mice

Specific pathogen-free female BDF_1 (C57BL/6×DBA/2) and CDF_1 (BALB/c×DBA/2) mice were purchased from Charles River Japan, Inc. (Kanagawa). They were maintained in a barrier system and fed sterilized pellets and water *ad libitum*. They were 8~12 weeks old at the start of each experiment.

Tumors

L1210 leukemia cells which had been maintained intraperitoneally in CDF_1 mice by weekly passage at Institute of Microbial Chemistry for more than 10 years were used for the present study.

Forphenicinol and Other Reagents

Forphenicinol was synthesized by Banyu Pharmaceutical Co., Ltd. according to the methods re-

ported by MORISHIMA *et al.*[5]. Anti-I-A^d (No. 1360) and FITC-F(ab')goat-anti-mouse IgG (No. 1311-0111) were purchased from Becton Dickinson Monoclonal Center, Inc. (Mountain View, California) and Cappel Laboratories, Inc. (West Chester, Pennsylvania), respectively. Glutaraldehyde was obtained from Tokyo Kasei Kogyo Co., Ltd. (Tokyo), and concanavalin A (Con A, type IV) was from Sigma Chemical Co. (St. Louis, Missouri).

Identification of Ia-positive Macrophages

The immuno-fluorescence method reported by BELLER *et al.*[8] was used for identification of Ia-positive macrophages. Peritoneal cells taken from mice were suspended in phosphate-buffered saline (PBS) at a density of 5×10^5 cells/ml. Two ml of peritoneal cell suspension was placed into each of several 35-mm culture dishes containing a glass coverslip and incubated at 37°C for 1 hour. After removal of non-adherent cells, the adherent cells, most of which were morphologically judged to be macrophages, were incubated with or without 1.5 ml of a 400-fold dilution of antimouse I-A^d solution at 4°C for 30 minutes, after which the coverslip was washed and then exposed to an 80-fold dilution of FITC-F(ab')$_2$goat-anti-mouse IgG solution at 4°C for another 30 minutes. The degree of dilution for both of these antibodies was determined by preliminary experiments. Finally, the coverslip was thoroughly washed with PBS and then observed under epilumination with a fluorescence microscope. Cells with a fluorescence ring were counted as Ia-positive cells.

Preparation of L1210 Vaccine

L1210 vaccine was prepared by the method described by KATAOKA *et al.*[7] with a slight modification. L1210 cells suspended in PBS at a density of 1×10^7 cells/ml were mixed with the same volume of PBS containing 0.05% glutaraldehyde, incubated at 4°C for 1 hour, and subsequently exposed to 165 μg/ml of Con A at 4°C for another 1 hour. After being washed, the cells were used as L1210 vaccine.

Results

Effect of Forphenicinol on the Generation of Ia-positive Macrophages in Non-immune Mice

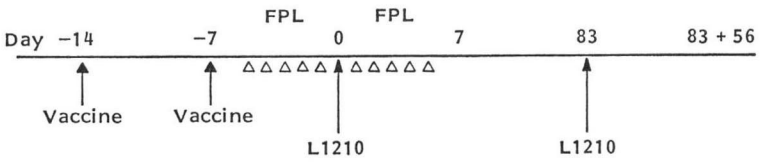
Healthy BDF$_1$ mice received oral administration of 0.5 mg/kg forphenicinol once a day for 5 days (day 1~5). Peritoneal cells were taken from mice on day 2, 4 and 7 and examined for the number of Ia-positive macrophages. The effect of forphenicinol on Ia-positive macrophages in mice implanted with 1×10^5 L1210 cells 1 day before the start of the 5-day drug treatment was also examined. As shown in Table 1, on day 4 and 7 the percentage of Ia-positive peritoneal macrophages in healthy mice given forphenicinol was almost triple of that in control mice. In the absence of forphenicinol the number of Ia-positive macrophages was markedly reduced by the implantation of a relatively large number of

Table 1. Effect of forphenicinol (FPL) on the production of Ia$^+$-macrophages in healthy BDF$_1$ mice and in these implanted with L1210.

Tumor	FPL mg/kg, po	Percent of Ia$^+$-macrophages (±SD)*		
		Day 2	Day 4	Day 7
—	0	3.0±1.0		
—	0.5	2.3±1.8	9.3±4.3	8.7±4.4
+	0	0.2±0.1	1.0±0.7	0.6±0.3
+	0.5	3.0±2.5	2.8±3.2	0.9±0.8

* No. of Ia$^+$-macrophages/No. of adherent cells.
Mice: BDF$_1$ female, 12 weeks old, $n=3$.
Tumor: 1×10^5 cells/mouse ip on day 0.
FPL: Given on day 1~5.
Assay: Peritoneal macrophages+anti-I-A^d+FITC-F(ab')$_2$goat-anti-mouse IgG.

Table 2. Antitumor effect of FPL in combination with L1210 vaccine on L1210 leukemia in BDF_1 mice.



FPL, po		Survival days*		P**	1st Challenge survivors on day 83	2nd Challenge survivors on day 83+56
Administration (day)	Dose (mg/kg)	Mean	T/C (%)			
—	—	17.0	100		0/4	—
—	—	—	—		—	0/4
−5～−1	0.01×5	33.8	199		1/5	1/1
〃	0.1 ×5	26.2	154		1/5	1/1
〃	1.0 ×5	36.2	213		2/5	2/2
1～5	0.01×5	60.0	357	<0.001	4/4	3/4
〃	0.1 ×5	44.2	260	<0.05	3/5	1/3
〃	1.0 ×5	16.5	97		0/4	—

* Calculated on day 60.
** P Student's t-test.
Mice: BDF_1 female, 8 weeks old.
Vaccine: Glutaraldehyde-Con A-treated L1210, 1×10^6 cells/mouse, ip.
Tumor: 1st Challenge, 1×10^2 cells/mouse, ip. 2nd Challenge, 1.5×10^2 cells/mouse, ip.

L1210 cells (1×10^5), whereas administration of the drug suppressed this reduction of Ia-positive macrophages up to 4 days after the tumor implantation.

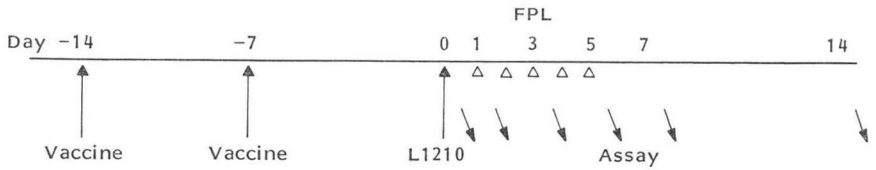
Antitumor Effect of Forphenicinol in Combination with L1210 Vaccine on L1210 Leukemia in Mice

BDF_1 mice were injected with 10^6 L1210 vaccine cells/mouse, followed by a second identical inoculum 1 week later, and then implanted intraperitoneally with 10^2 L1210 cells/mouse 1 week after the second vaccination. They were given 0.01, 0.1, or 1.0 mg/kg of forphenicinol orally once a day for 5 consecutive days before or after the tumor implantation. As shown in Table 2, both treatments prolonged the survival period in the mice; in the case of post-treatment with 0.01 and 0.1 mg/kg forphenicinol, the T/C (%) calculated in each case 83 days after the challenge of L1210 was more than 260% ($P<0.05$ by Student's t-test); and the mice had acquired concomitant immunity to L1210 since they survived a second L1210 challenge.

Effect of the Drug on the Generation of Ia-positive Macrophages in Mice Vaccinated with L1210 Vaccine Prior to Implantation with L1210

BDF_1 and CDF_1 mice were given L1210 vaccine as described above. Forphenicinol was given by oral administration at 0.5 mg/kg once a day for 5 days (day 1～5) starting 1 day after the tumor implantation. As shown in Table 3, vaccination scarcely enhanced the generation of Ia-positive macrophages in healthy mice (both BDF_1 and CDF_1). Implantation with 10^2 L1210 cells caused less change in the generation of Ia-positive cells than the implantation with 10^5 cells which was shown in Table 1. The generation of Ia-positive macrophages in mice without vaccination was almost the same as that in con-

Table 3. Effect of FPL on the population of Ia$^+$-macrophages in mice given L1210 vaccine prior to implantation with L1210.



Mouse	Vaccine	Tumor	FPL mg/kg, po	Percent of Ia$^+$-macrophages (mean±SD)						Days of survival (mean)
				Day 0	Day 1	Day 3	Day 5	Day 7	Day 14	
BDF_1	—	—	0	2.6±1.0	2.9±2.0	3.1±1.1	2.0±0.7	2.5±0.7	2.2±1.2	—
〃	+	—	0	3.0±0.6	3.7±1.5	3.6±0.7	4.0±1.9	2.6±0.7	1.9±0.7	>30.0
〃	—	+	0	NT	3.9±0.3	2.5±0.6	4.3±2.2	1.6±0.8	0.0	17.7
〃	+	+	0	NT	5.5±1.0*	2.9±0.8	3.0±0.9	2.7±0.9	0.0	19.1
〃	+	+	0.5	NT	NT	4.8±0.1*	10.1±0.4*	5.1±0.6*	0.0	25.4**
CDF_1	—	—	0	3.1±0.5	2.7±1.9	4.3±2.2	3.1±1.0	1.6±0.8	3.2±1.1	—
〃	+	—	0	4.5±1.6	5.3±0.3	4.0±1.1	4.1±1.2	3.9±0.6	3.0±0.5	>30.0
〃	—	+	0	NT	2.9±2.3	3.9±0.9	4.5±0.3	3.0±0.8	0.0	16.3
〃	+	+	0	NT	5.1±1.5	5.1±1.2	6.0±1.4	3.2±1.4	0.0	17.3**
〃	+	+	0.5	NT	NT	4.1±2.8	5.4±0.8	5.6±1.2	0.0	17.9

$P<0.05$ by Student's t-test (*) and by U-test (**) with respect to each control, calculated on day 30. Mice: BDF_1 and CDF_1 female, 8 weeks old.
Vaccine: Glutaraldehyde-Con A-treated L1210, 10^6 cells/mouse.
Tumor: L1210, maintained ip in CDF_1 mouse, 100 cells/mouse, ip, on day 0.
Assay: Mϕ+anti-I-A^d+FITC-F(ab′)$_2$goat-anti-mouse IgG.

trols until 7 days after the tumor implantation. However, Ia-positive peritoneal macrophages disappeared by 14 days after the implantation. It is noteworthy that the generation of Ia-positive macrophages in vaccinated-BDF_1 mice was enhanced by the implantation of tumor cells 1 day before ($P<0.05$), while the tumor implantation did not influence the generation of Ia-positive macrophages in vaccinated CDF_1 mice.

On the other hand, when 0.5 mg/kg forphenicinol was given orally to mice which had been vaccinated and then implanted with tumors, the number of Ia-positive macrophages in the BDF_1 mice increased 3-fold by day 5, and these mice survived longer. However, these effects of forphenicinol were not shown in the CDF_1 mice.

Discussion

The generation of Ia-positive macrophages was suppressed in BDF_1 mice implanted with 10^5 L1210 cells. And when they were implanted with 10^2 L1210 cells, with growing tumors, Ia-positive macrophage generation was reduced after a transient increase[8,9]. Considering the important role of Ia-positive macrophages in immune response, it is of great importance that forphenicinol induces the generation of Ia-positive macrophages in healthy mice and, furthermore, that it has the ability to restore the generation of Ia-positive macrophages which had been reduced by tumor burden.

Vaccination with 10^7 glutaraldehyde-Con A-treated L1210 cells markedly increased the survival time of both BDF_1 and CDF_1 mice implanted with 10^2 L1210 cells, and oral administration of forphenicinol increased the survival period of L1210-bearing BDF_1 mice in combination with L1210 vaccine which had been prepared from L1210 cells maintained in the peritoneal cavity of BDF_1 mice. However, the drug was ineffective in prolonging the survival of L1210-bearing CDF_1 mice vaccinated with the same vaccine. And Ia-positive macrophage generation was stimulated by forphenicinol in BDF_1, but not in CDF_1 mice. Therefore we are convinced of a neat set of correlations among the appearance of Ia-antigens, the life-span of tumor-bearing mice, and the effect of forphenicinol. The specificity in mouse strain also suggests that forphenicinol augments so-called F_1 effect on L1210 cells[10~12].

From the present studies, we suggest that a preferential induction of Ia-positive peritoneal macrophages may be one of the major effects of forphenicinol on the host defense system in mice.

Acknowledgments

We thank Dr. T. KATAOKA, Japanese Foundation for Cancer Research, for helpful discussion and advice.

References

1) 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
2) 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
3) ISHIBASHI, T.; Y. HARADA, M. TAKAMOTO & A. SHINODA: Immunopotentiation with forphenicinol: Increased resistance to *Pseudomonas* septicaemia in mice. J. Antibiotics 38: 430~432, 1985
4) 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
5) MORISHIMA, H.; J. YOSHIZAWA, R. USHIJIMA, T. TAKEUCHI & H. UMEZAWA: Synthesis of forphenicinol and forphenicine. J. Antibiotics 35: 1500~1506, 1982
6) BELLER, D. I.; J.-M. KIELY & E. R. UNANUE: Regulation of macrophage populations. I. Preferential induction of Ia-rich peritoneal exdates by immunologic stimuli. J. Immunol. 124: 1426~1432, 1980
7) KATAOKA, T.; F. OH-HASHI, S. TSUKAGOSHI & Y. SAKURAI: Induction of resistance to L1210 leukemia in BALB/c × DBA/2Cr F_1 mice, with L1210 cells treated with glutaraldehyde and concanavalin A. Cancer Res. 37: 964~968, 1977

8) TAYLOR, D. D. & P. H. BLACK: Inhibition of macrophage Ia antigen expression by shed plasma membrane vesicles from metastatic murine melanoma lines. J. Natl. Cancer Inst. 74: 859~867, 1985
9) STEEG, P. S.; H. M. JOHNSON & J. J. OPPENHEIM: Regulation of murine macrophage Ia antigen expression by an immune interferon-like lymphokine: inhibitory effect of endotoxin. J. Immunol. 129: 2402~2406, 1982
10) SHEARER, G. M. & G. CUDKOWICZ: Induction of F_1 hybrid antiparent cytotoxic effector cells: An *in vitro* model for hemopoietic histoincompatibility. Science 190: 890~893, 1975
11) NAKANO, K.; I. NAKAMURA & G. CUDKOWICZ: Generation of F_1 hybrid cytotoxic T lymphocytes specific for self H-2. Nature 289: 559~563, 1981
12) REIMANN, J. & R. G. MILLER: Generation of autoreactive cytotoxic T lymphocytes under limiting dilution conditions. J. Immunol. 131: 2128~2134, 1983