# STUDIES ON ANTITUMOR ACTIVITY OF PRUMYCIN

## IV. EFFECT OF PRUMYCIN ON MOUSE IMMUNE SYSTEM*

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The effect of prumycin on the mouse immune system was studied. Prumycin at a dose of 75 mg/kg ($\frac{1}{2}LD_{50}$) showed essentially no suppression of hemolytic plaque forming cells (PFC) against sheep red blood cells (SRBC) when it was administered on day 0, 1 or 2. However, a weak suppression was observed by administration of prumycin on day 3. Also a remarkable suppression of PFC was demonstrated when mouse spleen cells were pre-incubated with prumycin at the concentration over 62.5 mg/kg *in vitro*. The delayed-type hypersensitivity reaction against SRBC was remarkably suppressed by cyclophosphamide, but it was not much by prumycin, except by administration on the day of elicitation. Prumycin of a high concentration inhibited an uptake of $^3$H-thymidine by mouse spleen lymphocytes incubated with or without phytohemagglutinin, but that of a low concentration, 1.9~0.03 mcg/ml, slightly increased the blastogenic stimulation of lymphocytes.

Prumycin[1,2] possesses potential antitumor activity against mouse mammary adenocarcinoma[3], and has the following characteristics.

1) The mechanism of action on tumour cells appears to be related to inhibition of protein and DNA synthesis[5].
2) High concentration was detected in the kidney, skin, uterus, bone, liver, lung and stomach, while the concentration in the brain, heart, spleen and testis was too low to detect[4].
3) It caused alopecia in mouse and rat, and vomiting in dog, but not bone marrow toxicity[3].

The suppression of immune response by anticancer agents is increasingly discussed as unfavorable side effect[10] on host resistance against cancer. Also for establishing immunochemotherapy of cancer, understanding of the characteristics of the immunosuppressive action of each chemotherapeutic agent is thought to be indispensable.

The present paper is a report of the study on the effect of prumycin on the humoral and cellular immunity in mice.

## Materials and Methods

### Animals

$CDF_1$ mice of about 20 g body weight were purchased from Shizuoka Agriculture Cooperative Association for Laboratory Animals.

### Chemicals

Prumycin was prepared as reported in the previous paper[3]. Mitomycin C (Kyowa Hakko Kogyo), cyclophosphamide (Shionogi Pharm. Co.) and gentamicin (Shionogi Pharm. Co.) were used as reference agents.

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Antigen and antibody assay

Sheep red blood cells (SRBC) were purchased from Japan Biotest Laboratories and were used as suspension of 20% concentration after washing with physiological saline three times. SRBC ($3 \times 10^8$ cells) were injected into mice intravenously, and antibody-forming cells (PFC) in the spleen after 4 days were measured by the method of CUNNINGHAM and SZENBERG[6].

Suppression of antibody production *in vitro*

The spleen cells of mouse, 4 days after sensitization with SRBC, were incubated at 37°C for 60 minutes with prumycin, gentamicin, or mitomycin C of various concentrations, and washed, then PFC was measured.

Test for delayed type hypersensitivity (DTH)

SRBC ($1 \times 10^5$ cells) were injected intravenously into mice according to the method of LAGRAGE *et al.*[7], $1 \times 10^8$ cells of the same antigen were injected into footpad after 4 days, and thickness of the footpad was measured after more 24 hours.

Assay of blastogenic stimulation of mouse spleen lymphocytes

Lymphocytes were separated from the mouse spleen cells by gradient centrifugation using the lymphocytes separating liquid (Japan Antibody Industries, Ltd.). Then lymphocytes were suspended at $1 \times 10^6$/ml in RPMI-1640 medium containing 5% fetal calf serum and $5 \times 10^{-5}$ M 2-mercaptoethanol, and 0.2 ml of the cell suspension was placed in each well of a microtest plate (Falcon). Prumycin or mitomycin C of various concentrations was added to each well with or without phytohemagglutinin (PHA) at the final concentration of 43 mcg/ml. After incubation at 37°C for 48 hours, 1 $\mu$Ci/well of $^3$H-thymidine (25 Ci/mmol, The Radiochemical Centre) was added, incubated for further 5 hours, the cells were harvested by mutiple cell harvester (Labo Science), and incorporation of $^3$H- thymidine was measured by a liquid scintillation counter.

## Results

### Effect of Prumycin on Antibody-forming Cells

In preliminary experiments it was observed that the number of antibody-forming cells (PFC) in the spleen of $CDF_1$ mice reaches a maximum 4 days after sensitization with $3 \times 10^8$ cells of SRBC. Therefore, 75 mg/kg ($\frac{1}{2}LD_{50}$) of prumycin was intraperitoneally administered into mice simultaneously with or 1, 2, or 3 days after sensitization, and PFC was measured 4 days after sensitization. Mitomycin C at the dose of 4.2 mg/kg ($\frac{1}{2}LD_{50}$) was administered 2 days after sensitization. As shown in Table 1, mitomycin C suppressed PFC remarkably, but prumycin showed little suppression except for its administration 3 days after sensitization.

Table 1. Effect of prumycin on antibody-forming cells in mouse spleen.

| Drugs | Time of administration[a] | on Day 4 | | | |
|---|---|---|---|---|---|
| | | PFC/$10^6$ spleen cells[b] | (%) | PFC/spleen ($\times 10^3$) | (%) |
| Control | — | 1,776±196 | (100) | 595±26 | (100) |
| Prumycin 75 mg/kg i.p. | Day 0 | 1,552±202 | ( 87) | 543±71 | ( 91) |
| | Day 1 | 1,818±251 | (102) | 601±13 | (101) |
| | Day 2 | 1,751±171 | ( 99) | 595±26 | (100) |
| | Day 3 | 1,155±301 | ( 65) | 386±84 | ( 65) |
| Mitomycin C 4.2 mg/kg i.p. | Day 2 | 110± 30 | ( 6) | 26± 7 | ( 4) |

a) $DCF_1$ mice were sensitized with $3 \times 10^8$ SRBC intravenously on day 0.
b) PFC was measured by the method of CUNNINGHAM *et al.*[6] on day 4.

### Effect of Prumycin on Antibody Production of Hemolytic Plaque Forming Cells *In Vitro*

The spleen cells of $CDF_1$ mouse 4 days after sensitization with SRBC were preincubated *in vitro* with prumycin, gentamicin or mitomycin C at 37°C for 60 minutes and then the residual PFC were measured. As can be seen from Table 2, prumycin suppressed PFC at concentrations higher than 62.5 mcg/ml, but gentamicin did not result in suppression even at a high concentration of 500 mcg/ml. Mitomycin C, which inhibits the growth of HeLa cells at a concentration 1/10 or lower of that of prumycin[5], showed no suppression of PFC at a concentration of 6.3 mcg/ml. No change in the cell viability was observed by the trypan blue dye exclusion test after incubation with prumycin (1,000 mcg/ml), gentamicin (2,000 mcg/ml), or mitomycin C (100 mcg/ml) at 37°C for 60 minutes.

### Effect of Prumycin on Delayed-type Hypersensitivity (DTH) Reaction

The effect of prumycin, mitomycin C and cyclophosphamide on mouse DTH is shown in Table 3. Cyclophosphamide showed strong suppression even by administration of 150 mg/kg ($<\frac{1}{2}LD_{50}$) 1 day after sensitization. Mitomycin C also showed a suppression by administration of 4.2 mg/kg ($\frac{1}{2}LD_{50}$) especially 1 day before sensitization and elicitation. However, 75 mg/kg ($\frac{1}{2}$ $LD_{50}$) of prumycin showed slight suppression except by administration

Table 2. Effect of prumycin on antibody production of hemolytic plaque forming cells *in vitro*.

| Cells treated with | Concentration (mcg/ml) | PFC/$10^6$ spleen cells | % |
|---|---|---|---|
| None | — | 1,063± 63 | 100 |
| Prumycin | 1,000 | 125± 31 | 12 |
| | 250 | 156± 26 | 15 |
| | 62.5 | 406± 29 | 38 |
| | 15.7 | 594± 30 | 56 |
| | 3.9 | 813±108 | 77 |
| Gentamicin | 2,000 | 750±107 | 71 |
| | 500 | 1,000±200 | 94 |
| | 125 | 1,032± 86 | 97 |
| Mitomycin C | 100 | 688± 31 | 65 |
| | 25 | 781± 84 | 74 |
| | 6.3 | 907± 30 | 85 |

Spleen cells from $CDF_1$ mice sensitized with $3\times10^8$ SRBC 4 days before were suspended into RPMI-1640 medium at the concentration of $20\times10^6$/ml and incubated with prumycin, gentamicin, or mitomycin C at 37°C for 60 minutes. After washing PFC was detected by the method of CUNNINGHAM *et al.*[6]

Table 3. Effect of prumycin on mouse delayed-type hypersensitivity reaction.

| Treatment | Dose mg/kg | Time (Day) | Foot pad swelling (1/100 mm) | % |
|---|---|---|---|---|
| Control | | | 96±10 | 100 |
| Prumycin | 10 | −2~+3 | 102± 2 | 106 |
| | 75 | −1 | 76± 8 | 79 |
| | 75 | 0 | 96±10 | 100 |
| | 75 | +1 | 80±22 | 83 |
| | 75 | +3 | 61± 8 | 64 |
| | 75 | +4 | 48±12 | 50 |
| Mitomycin C | 1 | −2~+3 | 77±13 | 80 |
| | 4.2 | −1 | 30±19 | 31 |
| | 4.2 | 0 | 59±19 | 62 |
| | 4.2 | +1 | 56±22 | 58 |
| | 4.2 | +3 | 19± 9 | 25 |
| | 4.2 | +4 | 48±10 | 50 |
| Cyclophosphamide | 150 | +1 | 14± 3 | 15 |

$CDF_1$ mice were sensitized with $1\times10^5$ SRBC intravenously on Day 0. Prumycin and mitomycin C were injected intraperitoneally into mice successively from day −2 to day 3, or once at various time. Then DTH were elicited with the injection of $1\times10^8$ SRBC into mouse footpad on day 4 and the increased thickness of footpad was measured 24 hours after the injection.

Table 4. Effect of prumycin on mouse lymphocytes blastogenic stimulation *in vitro*.

| Drugs | Concentration (mcg/ml) | Blastogenic stimulation | | | |
|---|---|---|---|---|---|
| | | −PHA (cpm) | % | +PHA (cpm) | % |
| Control | − | 3,038±239 | 100 | 24,432±2,283 | 100 |
| Prumycin | 30 | 353± 30 | 12 | 252± 111 | 1 |
| | 7.5 | 2,050±164 | 67 | 12,932± 548 | 49 |
| | 1.9 | 5,130±417 | 169 | 34,199±1,362 | 140 |
| | 0.47 | 5,033±967 | 168 | 31,578±1,013 | 129 |
| | 0.12 | 4,798±636 | 158 | 27,525±1,484 | 112 |
| | 0.03 | 3,979±872 | 131 | 26,579±1,116 | 109 |
| Mitomycin C | 0.1 | 1,500±278 | 49 | 16,323± 944 | 66 |
| | 0.025 | 2,281±289 | 75 | 21,495± 683 | 88 |
| | 0.006 | 3,351±454 | 110 | 22,854±2,250 | 94 |
| | 0.0016 | 4,335±262 | 143 | 24,344± 857 | 100 |
| | 0.0004 | 3,116±187 | 103 | 25,206± 998 | 103 |

Mouse spleen lymphocytes ($1 \times 10^6$/ml) were cultured with prumycin or mitomycin C, with or without PHA (43 mcg/ml) in volume of 0.2 ml per well at 37°C for 48 hours, then 1 $\mu$Ci of $^3$H-thymidine per well was added and incubated for more 5 hours. Cells were harvested and incorporated of $^3$H-thymidine was measured by a liquid scintillation counter.

on the day of elicitation.

### Effect of Prumycin on Blastogenic Stimulation of Mouse Spleen Lymphocytes *In Vitro*

The effect of prumycin and mitomycin C on blastogenic stimulation of mouse lymphocytes is shown in Table 4. Prumycin suppressed incorporation of $^3$H-thymidine by lymphocytes remarkably at concentrations 7.5 mcg/ml or higher in both cases of PHA presence and absence, but it showed rather to stimulate slightly in both cases at concentrations between 0.12 and 1.9 mcg/ml.

## Discussion

It has been reported[8] that prumycin shows no decrease in peripheral white blood cells nor bone marrow toxicity. Suppression of the immune response by prumycin was anticipated to be weak, if any. In the present study, it was demonstrated that prumycin shows very slight suppression of antibody forming system to SRBC, except in the case of administration 3 days after sensitization. However, a suppression of PFC was observed when cells that had already been producing antibody were incubated *in vitro* with prumycin, although no change was observed in the viability of the cells. As reported in previous paper[5], prumycin strongly inhibits protein synthesis of HeLa S-3 cells, so that the suppression of PFC after incubation *in vitro* seems to be caused by inhibition of the antibody protein synthesis itself. The slight suppression of PFC by administration of prumycin 3 days after sensitization may also be considered to be caused by an inhibition of antibody protein synthesis of spleen lymphocytes, or that prumycin inhibited proliferation of antibody forming cells.

On the other hand, it is known that sensitized lymphocytes release lymphokine such as macrophage migration inhibitory factor (MIF), that is assumed to be a glycopeptide[8], by stimulation with antigen in the process of DTH reaction.

Geczy *et al.*[9] demonstrated that an antibody against lymphokine suppressed DTH reaction. Also Mizoguchi *et al.*[12] studied the time of inhibition of MIF release from sensitized lymphocytes by inhibitors of nucleic acid and protein syntheses *in vitro* and demonstrated that puromycin, in-

hibitor of protein synthesis, suppressed MIF strongly when it is added a few hours after lymphocytes stimulation by antigen, while mitomycin C when it is added prior to stimulation by antigen or in the initial stage. In the present *in vivo* study, prumycin suppressed DTH reaction most strongly when it was administered on the eliciting day rather than days before elicitation. Therefore, the suppression might be caused by the inhibition of lymphokine production in sensitized lymphocytes, although as a matter of course, a possibility of other mechanism is conceivable.

Addition of prumycin to incubated mouse lymphocytes suppressed incorporation of $^{3}$H-thymidine at concentration of 7.5 mcg/ml or higher, but slightly stimulated at 1.9 mcg/ml or lower concentrations.

It would also be of interest to investigate the effect of prumycin on the cyclic nucleotide level in the lymphocytes in view of the relationship between lymphocytes immune reaction and the cyclic AMP and GMP levels in the cells being indicated[11].

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