IMMUNOLOGICAL STUDIES ON SPORAMYCIN-TREATED ANIMALS

KANKI KOMIYAMA, IWAO UMEZAWA, TAKEHISA AKIYAMA* and TOJU HATA

Kitasato Institute, Shirokane, Minato-ku, Tokyo 108, Japan
*Kitasato University, School of Medicine
Kitasato, Sagamihara-shi, Kanagawa Prefecture, Japan

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Sporamycin showed a remarkable tumor regressive activity against sarcoma-180 with a single 5 mg/kg dose of intravenous administration. This antitumor effect on tumor and host animals was examined immunologically. As the results:

(1) When sarcoma-180 tumor cells were used as an antigen macrophage migration inhibition reaction by spleen cells derived from the tumor-bearing mice treated with sporamycin was positive at day 7~14 after the medication and was negative thereafter.

(2) The delayed hypersensitivity tested by the foot-pad reaction was positive in tumor-bearing mice treated with sporamycin, and no decrease of foot pad reaction was observed, whereas this reaction decreased remarkably in non-treated tumor-bearing mice.

(3) Sarcoma-180 tumor cells were mixed with spleen cells derived from sporamycin-treated mice, and were inoculated into normal *dd* mice. The growth of tumor cells was inhibited markedly, but no inhibition of tumor growth was observed in case of spleen cells derived from non-treated tumor bearing mice.

(4) Combined treatment of sporamycin with PS-K, an immunopotentiator, showed a remarkable synergistic effect.

Sporamycin is a new polypeptide antitumor antibiotic isolated from the culture of *Streptosporangium pseudovulgare,* strain No. PO-357[1,2]. This antibiotic has a significant antitumor activity in animal species such as mice bearing EHRLICH ascites carcinoma, L-1210 or P-388 leukemia, and sarcoma-180 solid tumor[3]. Sporamycin primarily inhibits DNA synthesis and caused strand scission of cellular DNA, but RNA and protein syntheses were not strongly affected in HeLa S-3 cells[4]. As for the antitumor activity of sporamycin[5], two activities are considered; one is a direct cytotoxic action in HeLa cells (*in vitro*) and the other is an indirect, host-mediated action in animal experiment (*in vivo*).

Experiments reported herein were designed mainly to elucidate the host-mediated activity and immunological activity of sporamycin, and the effect of sporamycin in combination with known immunopotentiators was also examined.

Materials and Methods

Animal and Tumor

Male *ddY* mice weighing 23~25 g obtained from Shizuoka Agricultural Cooperative Association, Hamamatsu, were used for the experiment. Sarcoma-180 (S-180) solid tumor was obtained from Takeda Chemical Industries, Ltd., Osaka, in 1961 and has been maintained in *ddY* mice by serial subcutaneous transplantation.

For the tumor inoculation, a small piece of tumor tissue was removed aseptically into EAGLE's minimum essential medium, and trimmed of necrotic and fibrous tissues. Cell suspension was prepared by forcing the tumor tissue through an 80-mesh stainless steel screen, and 1×10^5 cells were inoculated subcutaneously into the axillary region of *ddY* mice.

Chemicals

Sporamycin was purified in our laboratory according to the method reported previously[2]. PS-K (Kureha Chemical Ind., Tokyo), Broncasma berna (Sanwa Kagaku Kenkyusho Co., Ltd., Nagoya), levamisole (Kyowa Hakko Kogyo Co., Ltd., Tokyo), and OK-432 (Chugai Pharmaceutical Co., Ltd., Tokyo) were kindly supplied.

Macrophage Migration Inhibition Test

Spleen cells: Groups of 7 mice bearing S-180 were injected with a single 5 mg/kg dose of sporamycin i.p. on day 3 after tumor inoculation. The mice were sacrificed on day 7, 14, 21, or 28 after the medication, and the spleen was excised. The spleen cells were used as sensitized lymphoid cells.

Macrophages: Peritoneal exudate cells obtained from guinea pigs injected intraperitoneally 10~14 days previously with liquid paraffin were used as macrophages.

Antigen: Ascites cells of S-180 was withdrawn from the peritoneal cavity of *ddY* mice, and the cells were washed 3 times with phosphate-buffered saline. Resulting cell suspension was sonicated for 4 minutes and centrifuged at 700 rpm for 1 minute. The supernatant was used as the antigen.

Culture Solution: As the culture medium, HANKS' BSS containing 20% normal calf serum, 0.5% lacto-albumin hydrolysate, 100 units penicillin G/ml, 100 μg dihydrostreptomycin and 0.0125 ml of 10% sodium carbonate solution per ml, pH 6.5~6.7 was used.

The spleen cells and macrophages were mixed at a ratio of 1 : 2 in HANKS' lacto-albumine solution. The resulting cells mixture was then packed into small glass capillary tubes. Capillary tubes filled with the cells were attached to the bottom of a glass chamber with silicone grease and added culture solution with or without the antigen. The chamber was incubated overnight at 37°C in 5% CO_2 incubator.

The area of the macrophage migration was expressed by the product of the transverse and longitudinal axes of the fun like migratorycircules under an inverted binocular microscope.

Macrophage migration inhibition was expressed by a % migration index (MI). This % index was calculated by following formula.

$$\% \text{ Migration index} = \frac{\dfrac{\text{AMA in group A with antigen}}{\text{AMA in group B with antigen}}}{\dfrac{\text{AMA in group A without antigen}}{\text{AMA in group B without antigen}}} \times 100$$

AMA: average migration area

A: mixture of spleen cells with macrophages in capillary

B: macrophage alone

Empirically[6] % migration index of below 95(%) was considered as positive.

Delayed Hypersensitivity Test

Groups of 7 mice bearing S-180 were treated with 5 mg/kg of sporamycin intravenously on day 3 after tumor inoculation. On days 7, 11 and 16 after drug administration, 0.1 ml of 5% sheep red blood cells as an antigen was injected subcutaneously. Four days later, one foot-pad was injected with 0.02 ml of the antigen and the opposite foot-pad was injected with the same volume of saline as the control. The foot-pad thickness of the two hind feet of individual mouse was measured under a stereomicroscope with a scale in the eye-piece just before and 24 hours after injection of the antigen. Increase in foot-pad thickness = (24A − 0A) − (24S − 0S), where A and S are thickness of foot-pad given antigen and saline, respectively, and 0 and 24 are time just before or 24 hours after the injection.

Neutralization Test

Neutralization test was performed according to WINN's method[7] modified partially as follows:

Mouse bearing S-180 tumor was injected with a single 5 mg/kg dose of sporamycin intravenously on day 3 after tumor inoculation. Fourteen days after the administration, the mouse was sacrificed, and the spleen was excised. Cell suspension was prepared by forcing the spleen through a 200-mesh stainless steel screen. The cells were washed and resuspended in MEM (1×10^7 cells/ml). Two ml of the suspension was placed in a Petri dish (2.5 cm in diameter), added with 1 ml of the suspension of tumor cells (5×10^5), and incubated at 37°C for 3 hours in 5% CO_2 incubator. After incubation, the mixture was centrifuged, resuspended in 0.5 ml of MEM, and 0.2 ml of this suspension containing 2×10^5

tumor cells and 4×10^6 spleen cells was inoculated into the axillary region of *dd* mice subcutaneously. The tumor growth was observed with the lapse of time.

Combined Treatment with Sporamycin and Immunopotentiators

Groups of 7 mice bearing 3-day-old S-180 solid tumor were given i.v. with a single 1.25 mg/kg dose of sporamycin, and then were injected OK-432, PS-K, Broncasma berna, or levamisole as an immunopotentiator, daily from days 4～11 after tumor inoculation, respectively, and effectiveness of the combination treatment was examined.

Experimental Results

Antitumor effect of sporamycin on S-180 tumor in mice with a single injection of 5 mg/kg dose of the antibiotic is shown in Fig. 1. In spite of a single administration, a moderate tumor growth was observed during the first 2 weeks, but tumor regression began thereafter, and the tumor regressed completely 6 out of 7 treated mice after 4～5 weeks.

For explanation of this tumor regressive activity, immunological responsiveness of tumor-bearing mice treated with sporamycin was examined.

(1) Delayed hypersensitivity reaction

Delayed hypersensitivity reaction of tumor-bearing mice treated with sporamycin was determined by the foot-pad reaction. As shown in Fig. 2, the foot-pad thickness of non-treated mice decreased in accordance with tumor growth, while no decrease of foot-pad thickness was observed in the treated group at the beginning of the medication and then increase of foot-pad thickness was observed at day 21 after the treatment.

(2) Macrophage migration inhibition reaction

Mice bearing S-180 were given a single 5 mg/kg dose of sporamycin, and sacrificed with lapse of time. The spleen cells of each treated mouse and peritoneal macrophages of normal guinea pig were mixed and packed into capillary tubes and macrophage migration inhibition was tested, with the antigen prepared from S-180 tumor cells in a small glass chamber. As shown in Table 1, macrophage migration index(%) was positive (under 95%) by spleen cells derived from treated mice sacrificed at days 7 and 14 after the medication, but became negative at days 21 and 28.

Fig. 1. Antitumor effect of sporamycin on S-180 tumor.
A group of 7 mice was treated with a single 5 mg/kg of sporamycin i.p. on day 3 after tumor inoculation.

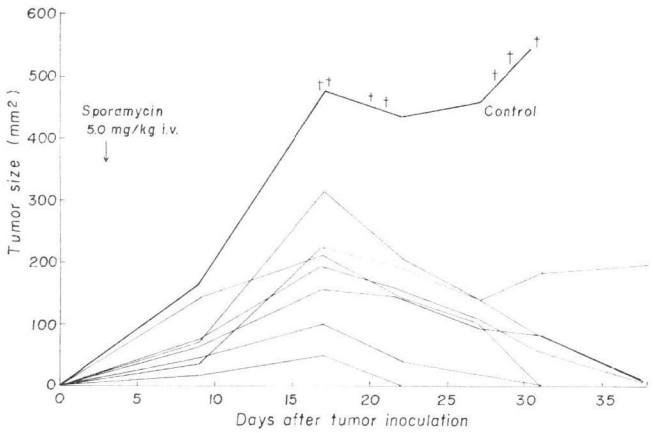
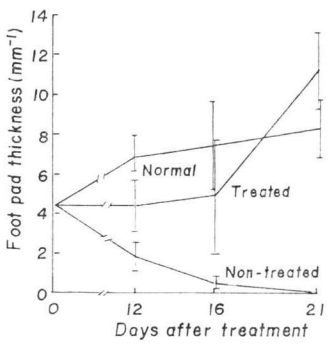


Fig. 2. Effect of sporamycin on delayed hypersensitivity in S-180 bearing mice.



(3) Neutralization test of tumor cells

Cell-mediated immune activity in mice treated with sporamycin was examined by a neutralization test using tumor cells and spleen cells derived from the treated mice.

S-180 tumor cells were mixed with spleen cells derived from mice treated with sporamycin 2 weeks before the experiment and incubated at 37°C for 3 hours. Then the mixture was inoculated into *ddY* mice subcutaneously. Antitumor effect was examined by the size of growing tumor and survival of the life span. As shown in Table 2, the size of tumor incubated with spleen cells derived from normal or non-treated tumor bearing mice increased remarkably with time, and no 60-day survivors were observed, whereas tumor growth was markedly inhibited by spleen cells derived from tumor bearing mice treated with sporamycin, and no tumor growth was observed in 8 of 12 mice on 60 days after the inoculation.

Table 1. Production of migration inhibitory factor from spleen cells derived from sporamycin-treated mice.

Days after treatment	No. of mice	Mean migration index (%)
Control	6	135±36.0
7	6	82± 8.9*
14	6	89±12.7*
21	6	126±16.1
28	6	106±13.9

* $P<0.05$

(4) Effect of sporamycin combined with immunopotentiator

Effect of sporamycin combined with several immunopotentiators such as OK-432, PS-K,

Table 2. Neutralization test of tumor cells by spleen cells derived from tumor-bearing mice treated with sporamycin.

Source of spleen cells	Donor No.	Recipient No.	Tumor size (mm^2) Day 15*	Mean	Day 27	Mean	60-day survivors
Normal mice (control)	1	1	812	397	900	616	0/4
		2	456		704		
	2	1	36		80		
		2	285		780		
Tumor-bearing mice, non-treated with sporamycin	1	1	169	273	285	415	0/4
		2	620		529		
	2	1	100		255		
		2	204		594		
Tumor-bearing mice, treated with sporamycin	1	1	—**	20	—	40	8/12
		2	—		—		
	2	1	—		—		
		2	—		—		
	3	1	—		—		
		2	120		255		
	4	1	—		—		
		2	—		—		
	5	1	36		100		
		2	—		—		
	6	1	16		81		
		2	72		+		

Spleen cells from each mouse (donor) and S-180 tumor cells were mixed and mixture was inoculated subcutaneously into two normal mice (recipient).

The tumor size was measured at days 15 and 27.

* Days after tumor inoculation.

** No tumor growth.

Table 3. Combination treatment with sporamycin and immunopotentiator.

Immuno-potentiator	Spora-mycin (mg/kg)	Dose/kg	Route	MSD*	ILS**	Cure rate
Control	—	—	i.v.	22	0	0/7
	2.5	—	i.v.	33	50	0/7
	1.25	—	i.v.	18	0	0/7
OK-432	—	80 KE	i.p.	24	9	0/7
	1.25	80 KE	i.p.	31	41	0/7
	1.25	40 KE	i.p.	22	0	0/7
PS-K	—	250 mg	i.p.	24	9	0/7
	1.25	250 mg	i.p.	>60	>173	5/7
	1.25	125 mg	i.p.	>60	>173	4/7
Broncasma berna	—	10 ml	i.p.	23	5	0/7
	1.25	10 ml	i.p.	25	14	0/7
	1.25	1 ml	i.p.	28	27	0/7
Levamisole	—	10 mg	s.c.	22	0	0/7
	1.25	10 mg	s.c.	35	59	0/7
	1.25	1 mg	s.c.	30	36	1/7

Administration schedule: Sporamycin: day 3 only, Other drugs: day 4~13

* MSD=Median survival day

** ILS=Increased life span

Broncasma berna or levamisole on S-180 in mice was tested. Three days after the inoculation of S-180, a single dose of 1.25 mg/kg of sporamycin was injected. From next day each agent was administered daily for 10 days. Each dose of test agents including sporamycin showed no antitumor activity when they were administered alone. As shown in Table 3, a remarkable effectiveness was observed with the combination of sporamycin and PS-K, and the tumor regressed completely in 4 or 5 out of 7 mice, but no combination effect was observed with OK-432, Broncasma berna or levamisole.

Discussion

Mouse transplantable tumor S-180 inoculated subcutaneously in mice showed a remarkable tumor regression effect with a single intravenous injection of sporamycin. According to histopathological findings of this effect reported by KAWAKUBO *et al.*[5], tumor cells were damaged by a direct cytocidal activity of sporamycin during a few day after the injection of the antibiotic, and thereafter this damage disappeared with the lapse of time. Infiltration of macrophages and lymphoid cells around the tumor and formation of granulation tissues were observed by approximately 2 weeks, and then majority of the tumor node regressed completely by about 30 days after the medication. From these findings it was considered that a host-mediated antitumor effect participates in the antitumor activity of sporamycin.

In our present experiments, the production of migration inhibitory factor from spleen cells derived from tumor-bearing mice treated with sporamycin was positive before tumor regression, and remarkable inhibition of tumor growth was observed when tumor cells were contacted with spleen cells derived from the mice treated with sporamycin before inoculation of tumor cells into normal mouse. Therefore, it is considered that spleen cells of tumor-bearing animal treated with sporamycin may have produced a tumor-inhibiting factor(s). Moreover, so far as the foot-pad test, sporamycin seemed to prevent immunosuppressive activity caused by tumor growth in mice and the host could easily acquire a resistance against tumor cells restored from the transient cytocidal activity of sporamycin.

In combined treatment of sporamycin with an immunopotentiator, a remarkable prolongation of survival time was observed when mice were treated with sporamycin plus PS-K but not with OK-432, Broncasma berna or levamisole at the present experimental conditions. PS-K was reported to prevent the immunosuppressive activity induced by antitumor agents or tumor growth in mice[8,9]. Though the mechanism of action of the synergistic effect between sporamycin and PS-K on S-180 tumor in mice is not known, the action of PS-K in this combination therapy appears to be different from above-mentioned immunopotentiators.

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