STUDIES ON A NEW ANTITUMOR ANTIBIOTIC, LARGOMYCIN. III

BIOLOGICAL PROPERTIES AND ANTITUMOR ACTIVITY OF LARGOMYCIN F-II

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Of the components of largomycin, largomycin F-II was biologically the most active. It is active against *Sarcina lutea* PCI 1001 at 12.5 mcg/ml, *Mycoplasma* species at 0.7 mcg/ml and HeLa S-3 cells at 0.1~0.5 mcg/ml. Largomycin F-II exhibited antitumor activity with a chemotherapeutic index of about 100 for ascites tumor and mouse leukemia after 7-day consecutive treatment with 0.4~0.8 mg/kg/day. Against solid tumors, however, largomycin F-II is less active. The high chemotherapeutic index could be explained by its selective toxicity to tumor cells compared with its toxicity to two diploid types of cell of primate origin.

Largomycin F-II, a new antitumor antibiotic, was isolated as a homogeneous powder from the culture filtrate of *Streptomyces pluricolorescens* MCRL-0367[1]. It is an acidic chromoprotein (molecular weight 25,000) with an isoelectric point at pH 4.2[2]. The present paper describes the biological properties of largomycin, and also the chemotherapeutic activity of largomycin F-II against several transplantable mouse tumors.

Materials and Methods

The sample:

The sample of largomycin F-II used was prepared from a culture filtrate and thorougly purified by repeated column chromatography with AE-cellulose and Sephadex G-100. It gave a single spot by polyacrylamide-gel disc electrophoresis and a symmetrical pattern on ultracentrifugation[2]. The samples of largomycin F-I and F-III used were prepared from the largomycin complex by gradient extraction with aq. ammonium sulfate solution.

Tissue-culture cells:

The tissue-culture cells used were as follows: (A) three systems of cells maintained in monolayer culture; 1) HeLa S-3 cells, 2) L cells, an established line of mouse fibroblast cells, and 3) cells of a fibroblast-like strain originated from a calf kidney but not established as a cell line, and (B) one system of cells maintained in floating culture; a clone of BURKITT lymphoma cells[3].

For toxicity tests, laromycin was incorporated in the appropriate maintenance medium in serial two-fold dilutions and inoculated into each tube culture. The culture media for cells and the toxicity profiles of cells to largomycin are given in the results section.

Experimental tumors:

EHRLICH ascites carcinoma (both ascitic and solid forms), Sarcoma-180 (ascitic form), and SN-36 leukemia were used for the present study. Inocula were taken from ascites of mice into which 5 million tumor cells had been inoculated intraperitoneally 5~7 days before. For the test, about one million ascitic tumor cells were inoculated intraperitoneally into each female mouse of the *dd* strain weighing approximately 20 g. In the case of the solid form of EHRLICH ascites carcinoma, 0.1 ml of the ascites containing one million tumor cells was implanted subcutaneously in the back of mice. For the survival test, 0.2 ~3.2 mg/kg of largomycin F-II was administered intraperitoneally once a day for 7 consecutive days starting from 24 hours after the tumor cell implantation. Mice which survived more than 50 days were described as survivors for 50 days.

Morphological changes of EHRLICH ascites carcinoma cells:

About 10^6 tumor cells were transplanted intraperitoneally to each mouse of the *dd* strain weighing 20 ± 2 g. On the 5 th day after transplantation one half of the tumor-bearing mice received either a single intraperitoneal injection of 1.8 mg/kg or 7.1 mg/kg of largomycin F-II dissolved in saline, and another half (the controls) received 0.5 ml of saline alone. A small amount of ascites was withdrawn from each animal immediately and at various intervals after injection of largomycin F-II. Smears of the ascites were stained with the MAY-GRUNEWALD-GIEMSA reagent and examined microscopically for cytological changes induced by largomycin F-II. At the same time, number of mitotic cells in each treated mouse were counted.

Toxicity test:

Acute toxicity tests were carried out at doses ranging from 20 to 50 mg/kg using 10 mice for each dose. Largomycin F-II was given intraperitoneally and the observation period was one week. LD_{50} was calculated by the LITCHFIELD-WILCOXON method. Subacute toxicity tests were carried out by intraperitoneal injections of either 6.4 or 1.6 mg/kg doses for 14 continuous days to 5 mice.

Experimental Results

Antimicrobial Test

The antimicrobial activitiy of largomycin determined by an agar-streak method are summarized in Table 1. The components were moderately active against a limited number of microorganisms such as *Sarcina lutea* PCI 1001 and *Corynebacterium xerosis*, but inactive against others at a concentration of 100 mcg/ml. Significant activity against *Mycoplasma* was noticed.

Cytotoxicity Test

Results of a cytotoxicity test in mammalian cell cultures are shown in Table 2. The minimum cytotoxic doses of largomycin F-II against cultured normal cells such as calf kidney or human embryo cells were almost 100 times those against HeLa S-3 or L cells. This suggested the possibilty of selective toxicity of largomycin to cancer cells. It is also interesting that the growth of BURKITT's lymphoma, one of the lymphoid cells, was completely inhibited by largomycin F-II at a concentration of 1 mcg/ml.

A concentration of 1,000 mcg/ml of largomycin F-II did not cause hemolysis of 2% rabbit red blood cells at 37°C.

Table 1. Antimicrobial activity of largomycin

Test organisms	Medium*	MIC (mcg/ml)		
		F-I	F-II	F-III
Staphylococcus aureus FDA-209P	I	>100	>100	>100
Staphylococcus aureus Terashima	I	>100	>100	>100
Streptococcus faecalis No. 3	I	>100	>100	>100
Sarcina lutea PCI-1001	I	50	12.5	12.5
Bacillus subtilis PCI-219	I	>100	>100	>100
Bacillus mycoides	I	50	12.5	12.5
Escherichia coli NIHJ	I	>100	>100	>100
Shigella flexneri 2b	I	>100	>100	>100
Salmonella typhosa	I	>100	>100	>100
Proteus vulgaris	I	>100	>100	>100
Pseudomonas aeruginosa	I	>100	>100	>100
Corynebacterium xerosis	I	50	6.25	6.25
Lactobacillus brevis	II	>100	>100	>100
Mycobacterium ATCC 607	III	>100	>100	>100
Xanthomonas oryzae	IV	>100	12.5	>100
Candida albicans 92	IV	>100	>100	>100
Aspergillus niger NIH	IV	>100	>100	>100
Mycoplasma pneumoniae Mac	V**	12.5	0.7	1.5
Mycoplasma pulmonis mA	V	25	0.7	1.5
Mycoplasma gallisepticum PG-31	V	25	6.25	12.5

* Medium I : Nutrient agar II : Tomato juice agar III : 1% Glycerol nutrient agar IV : SABOURAUD's agar
** The anti-mycoplasma activity of largomycin was determined by the agar plate diffusion assay method described by ARAI *et al.*[4]

Effect on Transplanted Tumors

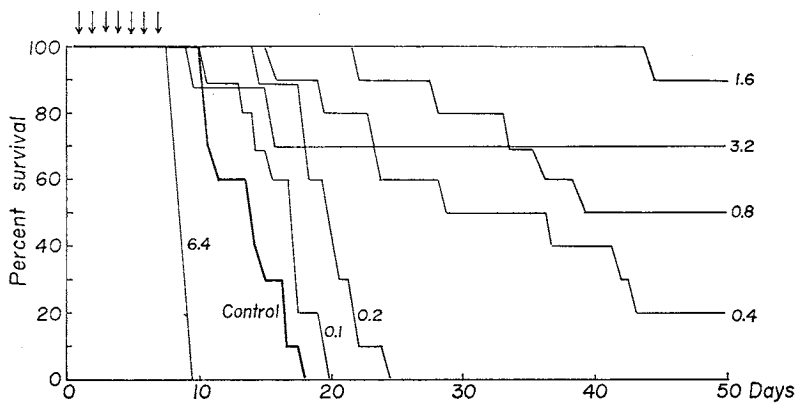
The results of experiments testing the effect of largomycin against the ascitic form of EHRLICH ascites carcinoma are illustrated in Fig. 1. When the treatment was started 24 hours after transplantation of the tumor cells, the increase of ascites was inhibited by daily doses of 0.4 mg/kg or higher, and the survival

Table 2. Toxicity of largomycin to mammalian cells

Cells	Minimum degeneration dose (mcg/ml)		
	F-I	F-II	F-III
HeLa S-3	5.0	0.1	1.25
L	1.0	0.25	0.5
H.E.K.*	100	20	100
Calf kidney	100	10	20
BURKITT's lymphoma	ND	1.0**	ND

* Human embryo kidney
** Minimum inhibitory concentration

Fig. 1. Effect of largomycin F-II on EHRLICH ascites carcinoma
(Administration : i. p., Dose : mg/kg/day)



period of tumor-bearing mice was markedly prolonged. Toxic signs appeared at a dose of 6.4 mg/kg/day.

As shown in Fig. 2, in mice inoculated with the ascitic form of Sarcoma-180, largomycin F-II showed a marked prolongation of the survival period at daily doses of 0.4 mg/kg and higher. With 1.6 mg/kg/day dose, the ascites was not increased and 90 % of the treated mice were survived for 50 days after inoculation.

Fig. 2. Effect of largomycin F-II on Sarcoma-180 (ascites)
(Administration : i. p.., Dose : mg/kg/day)

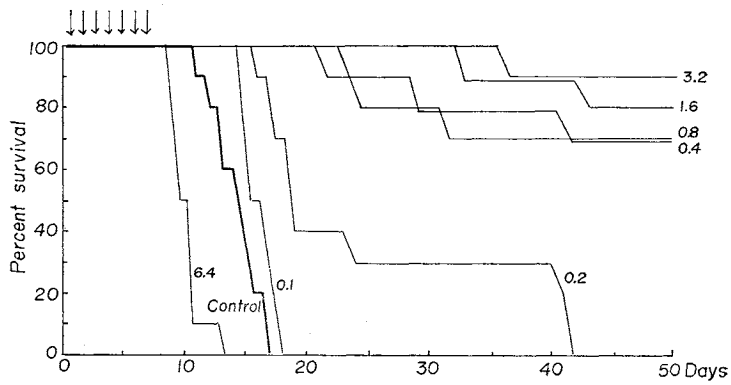
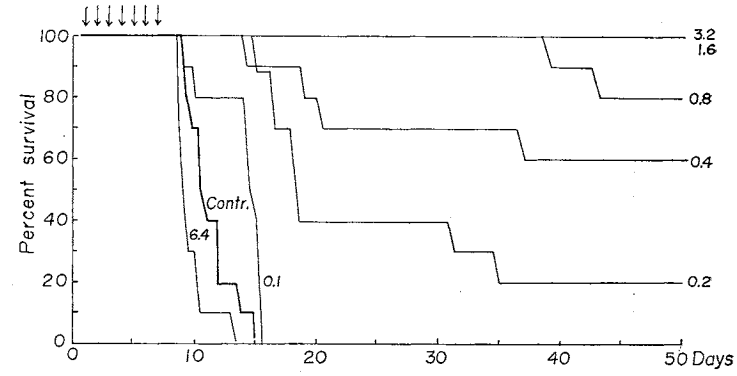


Fig. 3. Effect of largomycin F-II on SN-36 Leukemia
(Administration : i. p., Dose : mg/kg/day)



The effect on the ascitic form of SN-36 leukemia is given in Fig. 3. The survival period of tumor-bearing mice was markedly prolonged at a daily dose of more than 0.4 mg/kg. Especially noteworthy was the suvivors for more than 50 days of all mice received intraperitoneal administration of 3.2 mg/kg/day and 1.6 mg/kg/day. However, a dose of 6.4 mg/kg/day was toxic to mice bearing EHRLICH ascites carcinoma and Sarcoma-180.

Table 3. Effect of largomycin F-II on EHRLICH ascites carcinoma (solid form)

Start	Dose (mg/kg/day)	No. of deaths	Body weight (g)			Tumor inhibition*	
			Before	After	Diff.	weight (g)	ratio (%)
24 hrs.**	6.4	3/10	23.2	20.1	−3.1	1.18	58.0
	3.2	0/10	22.3	24.3	+2.0	1.77	37.0
	1.6	0/10	22.4	25.6	+3.2	2.78	1.0
	Control	0/10	22.4	25.0	+2.6	2.81	
10 days***	6.4	1/10	24.1	23.7	−0.4	3.48	27.3
	3.2	0/10	23.2	24.3	+1.1	4.31	10.0
	Control	0/10	23.7	25.2	+1.5	4.79	

* Inhibition ratio : 1-(Average tumor weight of treated/no treated)×100
** Intraperitoneal injection was started 24 hours after transplantation of the tumor cells and continued daily for 7 days.
*** Intraperitoneal injection was started 10 days after transplantation of the tumor cells and continued daily for 7 days.

The effect on solid tumors is summarized in Table 3. The degree of inhibition of tumor growth was assessed by the measuring tumor weight 10 and 20 days after transplantation of the tumor cells: the inhibition ratio was about 60 % at the 6.4 mg/kg/day dose. In the treatment starting from 10 days after transplantation, a slight regression was observed at the same dose.

Morphological Changes in EHRLICH Ascites Carcinoma Cells

As shown in Fig. 4, a decrease in the number of tumor cells was found immediately after a single administration of largomycin F-II, and reached a minimum after 24~48 hours. However, at the 1.8 mg/kg dose, the number of tumor cells recovered up to 80 % of the initial value by 72 hours. The changes in mitotic figures caused by largomycin F-II are summarized in Table 4. The decrease in number of mitotic cells reached a minimum at 12 hours when a majority of cells were abnormal in that they showed the scattering, adhesion or aggregation, shortening or swelling, and ball formation in chromosomes at metaphase (Plate 1). In addition, tumor cells in the resting stage showed the following morphological changes: swelling and loss of stainability of nucleus, swelling, vacuolation, deformation and leakage of cytoplasm (Plate 2).

Table 4. Changes in number of mitotic cells caused by largomycin F-II

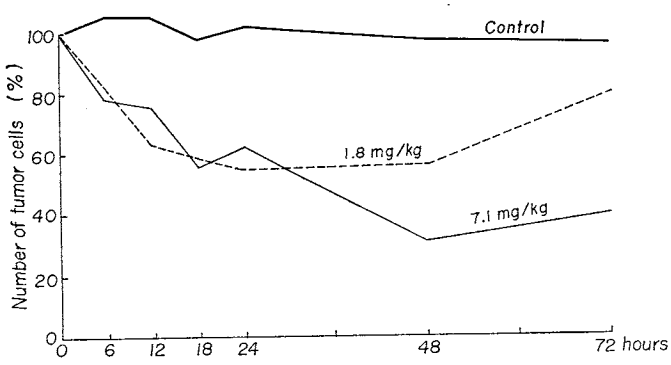
Does (mg/kg)	Mitotic phase	Number of mitotic cells (hrs. after treatment)						
		0	6	12	18	24	48	72
7.1	Pro-phase	23	2	1	4	3	8	7
	Meta-phase	26	16	7	10	11	12	28
	Ana-phase	8	5	2	2	1	5	4
	Telo-phase	10	7	0	0	0	0	2
	Total	67(2)	30(9)	10(13)	16(11)	15(9)	25(7)	44(10)
1.8	Pro-phase	21	12	1	5	6	19	20
	Meta-phase	26	23	11	10	30	4	20
	Ana-phase	10	4	3	4	2	8	8
	Telo-phase	11	2	2	1	2	12	6
	Total	68(1)	41(8)	17(11)	20(10)	40(13)	63(5)	54(6)

Largomycin F-II were injected intraperitoneally 5 days after inoculation of EHRLICH ascites carcinoma cells. Two thousands of the tumor cells for each animal were counted and the values of the number of normal mitotic cells recorded. Numbers in parentheses shows the number of abnormal mitotic cells.

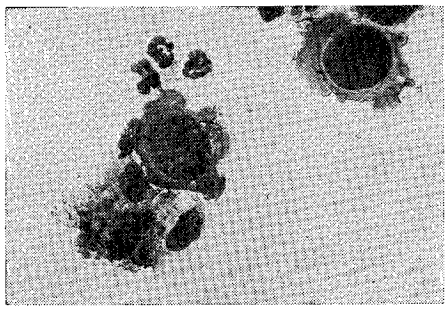
Toxicity of Largomycin F-II for Mice

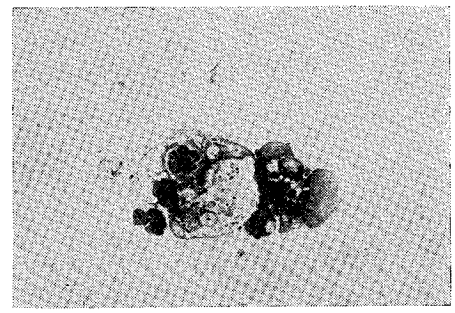
The LD_{50} in mice by the intraperitoneal route was determined to be 35.5 mg/kg, and a tendency for delayed toxicity was observed. In the consecutive injection group, the 6.4 mg/kg dose caused several signs of toxicity: mice began to weaken from the 5th day and then decrease

Fig. 4. Changes in number of EHRLICH ascites carcinoma cells (Administration : i. p.)



Plates 1 and 2. Morphological changes in EHRLICH ascites carcinoma cells with largomycin F-II. (i. p. 18 mg/kg) (×800×1/1.5)





of body weight, ruffled fur and mild diarrhoea followed by death within 15 days were observed.

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