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Studies on the effect of the tissue substance "cornin" on transplantable malignant tumors in mice

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Studies on the effect of the tissue substance "cornin" on transplantable malignant tumors in mice*

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Abstract

1. In the present experiments, Ehrlich ascites carcinoma (K-tsrain), JTC-11, and C3H mouse mammary tumor (A-strain) were used to study the inhibitory effects of two kinds of comins, crude muscle cornin and crude intestine comin. 2. Daily intraperitoneal administrations of both comins had shown a marked inhibitory effect on the Ehrlich ascites carcinoma. 3. Intestine comin was more effective on the inhibition of the growth of the Ehrlich ascites carcinoma than muscle cornin when administered intraperitoneally. 4. Daily subcutaneous adminstrations of muscle comin had no effect, but doses of 10 mg/mouse/day or 20 mg/mouse/day of intestine cornin had a slight or moderate inhibitory effect on the Ehrlich ascites carcinoma. 5. Intestine comin had an inhibitory effect on the growth of JTC-ll cells in vitro, and made the tumor cells to undergo morphological changes during incubation. 6. Daily intraperitoneal administrations of muscle comin had hardly any effect on the C3H mouse mammary tumor, but intestine comin was evidently effective in male. 7. Intraperitoneal administrations of intestine comin proved to be hardly effective on the C3H mouse mammary tumor, but only in the dose of 30 mg/ mouse/day, it had a moderate inhibitory effect in female. 8. Daily subcutaneous administrations of muscle comin had no effect on the C3H mouse mammary tumor, but intestine comin had a slight effect in male. 9. Muscle cornin had a slight or moderate effect on the C3H mouse mammary tumor, but intestine cornin was hardly effective in female when administered subcutaneously. 10. Repeated intraperitoneal administrations in doses of 30 mg/mouse/day of muscle comin produced intoxication in the treated mice. 11. In general, it seems that intestine comin is more effective on the inhibition of tumor growth than muscle comin.

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STUDIES ON THE EFFECT OF TISSUE SUBSTANCE "CORNIN" ON TRANSPLANTABLE MALIGNANT TUMOR IN MICE*

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INTRODUCTION

A tissue substance, discovered as a pupillo-contracting substance by NISIDA *et al*[23]. and later designated as "cornin" by HUKUI[12], was successfully extracted from several tissues including bovine cornea by alcoholic fractionation, and its biological actions and chemical properties were investigated by MIYAHARA[20], KADO[14] and TOKUMOTO[44]. In the course of studies on the biological action of cornin, HINO[10] and NISIDA *et al.*[24,25,26,27] demonstrated that cornin, extracted from either skeletal muscle of rabbit or bovine cornea, has an antimitotic action on the early development of sea urchin eggs. KANAO[15] and KOSHIMUNE[18] showed that cornin had an inhibitory effect on the incorporation of ^{32}P into regenerating rat liver and sea urchin eggs in the developmental stage. In addition, it was reported by NISIDA *et al*[28]. that the substance extracted from small intestine of mongrel dog (crude intestine cornin) had a significant inhibitory effect on the cell division of fibrosarcoma cells induced by DNA of SV40, but it revealed neither antimitotic nor degenerating effect on diploid fibrocytes of normal hamster and human embryonic cells.

The following experiments were designed to study the effects of cornin on the malignant cells *in vitro* and *in vivo*.

MATERIALS AND METHODS

Mice

The present experiments on Ehrlich ascites carcinoma were performed, using male ddN strain of mice, 6 to 7 weeks old, and weighing 22±2 g.

Zb strain of male and female mice, 8 to 10 weeks old and weighing 23±2 g (male) and 20±3 g (female), were used in the experiments on C3H mouse mammary tumor.

* An outline of this study was reported at the 44th General Meeting of the Physiological Society of Japan, Section III of the 17th General Assembly of the Japanese Medical Society in 1967 and at the 19th Chugoku-Shikoku Regional Meeting of the Physiological Society of Japan in 1967.

All were maintained on "Oriental" compressed diet (MF) and water ad libitum, and were kept in a room with constant temperature of $24 \pm 2°C$.

Tumor cells

Both the Ehrlich ascites carcinoma (K-strain), JTC-11, and the C3H mouse mammary tumor (A-strain) were kindly supplied by Professor SATO, Pathological Division, Cancer Institute of Okayama University Medical School, Okayama, 1966.

At the time of the first experiment, the Ehrlich ascites carcinoma was in its 16th transplant generation in mice after having been cultivated *in vitro* for 2020 days, 365 generations. Its transfer was done regularly at a 7-day intervals by the intraperitoneal inoculation.

The transfer of the C3H mammary tumor, over 80 generations in mice, was done every 21 days after the subcutaneous inoculation.

Inoculation of tumor cells

A donor mouse bearing 7-day old Ehrlich ascites carcinoma cells with a marked abdominal distension was selected at random and with the ascites removed aseptically with a 0.5 ml syringe having a 27 guage needle, and diluted with Crystal Violet solution the number of tumor cells was counted with a Buerker hemocytometer. These cells were inoculated intraperitoneally to recipient mice.

Tumors of 4 to 5 donor mice, having 21-day old C3H mouse mammary tumor, were expirpated and minced aseptically with a small scissors. The cell suspension in physiological saline solution, adjusted to contain 2.5×10^6 to 5.0×10^6 tumor cells in 0.25 ml, was inoculated subcutaneously to each mouse with a 0.5 ml syringe having a 22 guage needle.

Preliminary experiments

Various preliminary experiments on the Ehrlich ascites carcinoma were performed to determine the relation among the number of inoculated tumor cells, the viability and the body weight increase of the tumor-bearing mice[47].

1) Changes in the viability and body weight of the mice inoculated intraperitoneally with 7-day old ascitic fluid containing 1×10^7 tumor cells and with 14-day old ascitic fluid containing 2×10^7 tumor cells.

Each mouse of the first group was inoculated intraperitoneally with 7-day old ascites, the second group with 14-day old ascites. The results are shown in Fig. 1 (A : male, B : female).

In Fig. 1 (A) the body weight increased smoothly in both groups, but the range of all deaths in the second group was relatively wider than that in the first group. Therefore, it was decided to use 7-day old ascites in the subsequent

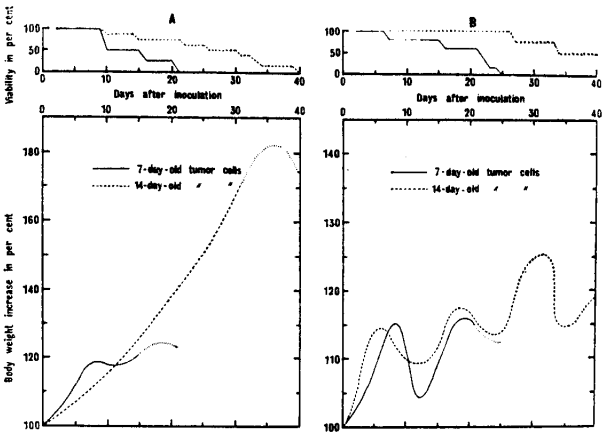

Fig. 1 Comparison of changes in the viability and body weight of the mice inoculated intraperitoneally with 7-day old and 14-day old Ehrlich ascites carcinoma cells.
A : male, B : female

experiments. On the other hand, in Fig. 1 (B), the body weight did not increase smoothly in both groups and spontaneous regression was observed in several mice in the second group.

2) Changes in the viability and body weight of male and female mice inoculated intraperitoneally with respective number of 7-day old ascitic tumor cells.

Twenty-four mice (12 males and 12 females) were divided into 3 groups. Each mouse of the first group was inoculated with 10^7 tumor cells, the second group 10^6 tumor cells, and the third group 10^5 tumor cells. The results of these studies are illustrated in Fig. 2 (A : male, B : female).

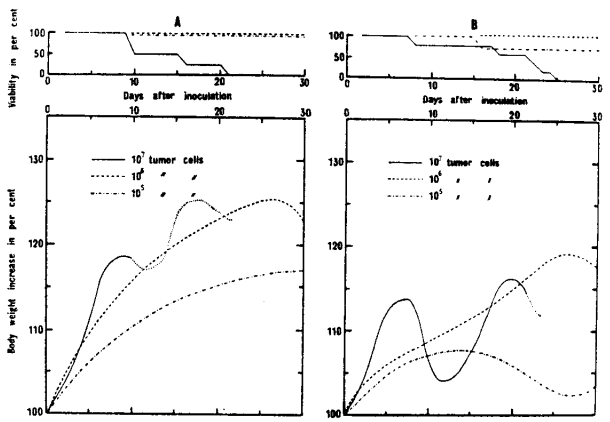

Fig. 2 Comparison of changes in the viability and body weight of male and female mice inoculated intraperitoneally with respective number of 7-day old Ehrlich ascites carcinoma cells.
A : male, B : female

The mice of the second and the third groups of both sexes survived over 30 days. On the other hand, the mice of the first group died within 1 to 3 weeks after inoculation and the body weight increased smoothly in male but not so in female. From the results of this experiment, it was considered the best procedure to be the intraperitoneal inoculation with 10^7 tumor cells to male mice.

3) Changes in the viability and body weight of the male mice of two groups maintained on two different diets.

The mice of the first group were given "Oriental" compressed diet (MF), and mixed diet (corn : wheal = 1 : 1) in the second group.

According to the result of this study shown in Fig. 3, all the deaths in the

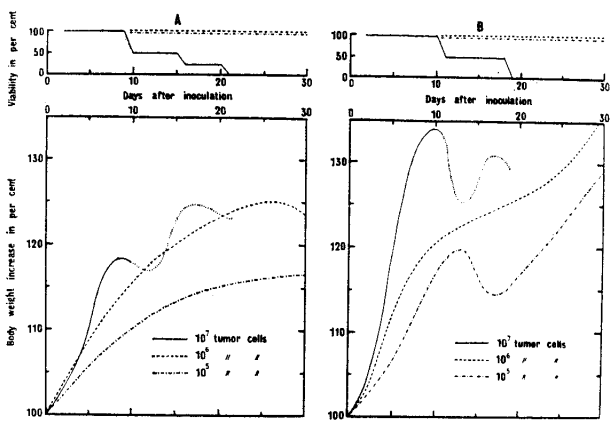

Fig. 3 Comparison of changes in the viability and body weight of the mice of the two groups maintained on two different diets.
A : mixed diet, B : compressed diet

first group occurred within rather small range and moreover, ascites developed more abundantly in the second group. Therefore, in the subsequent experiments mice were fed on compressed diet.

The mean survival time of 310 untreated mice inoculated intraperitoneally with 10^7 tumor cells of 7-day old Ehrlich ascites carcinoma was 13.6 ± 4.4 days.

Table 1 and Fig. 4 show the rate of tumor growth after subcutaneous inoculation with 2.5×10^6 C3H mouse mammary tumor cells.

Cornin

Two kinds of cornins, crude muscle cornin and crude intestine cornin, extracted by the same method as described in a previous paper[5], were used for antitumor screening with the Ehrlich ascites carcinoma and the C3H mouse mammary tumor. Muscle cornin was extracted from skeletal muscle of rabbit

Table 1 Rate of Tumor Growth after Subcutaneous Inoculation with 2.5×10^6 C3H Mouse Mammary Tumor Cells

(1) Male (16 mice)

Days after inoculation	Mean length of the two maximum perpendicular axes of the tumor (by calipers)		$ab^{3/2}$ (cm^3)	Tumor growth rate (%)
	a (cm)	b (cm)		
3	0.51±0.03	0.52±0.08	0.140	100
6	0.65±0.05	0.61±0.06	0.265	192
10	1.32±0.20	0.86±0.12	1.070	786
17	2.00±0.35	1.55±0.10	5.440	3994

(2) Female (16 mice)

Days after inoculation	Mean length of the two maximum perpendicular axes of the tumor (by calipers)		$ab^{3/2}$ (cm^3)	Tumor growth rate (%)
	a (cm)	b (cm)		
3	0.57±0.07	0.52±0.05	0.163	100
6	0.69±0.10	0.63±0.04	0.291	177
10	1.23±0.16	0.91±0.11	1.237	765
17	1.89±0.30	1.52±0.02	5.021	3215

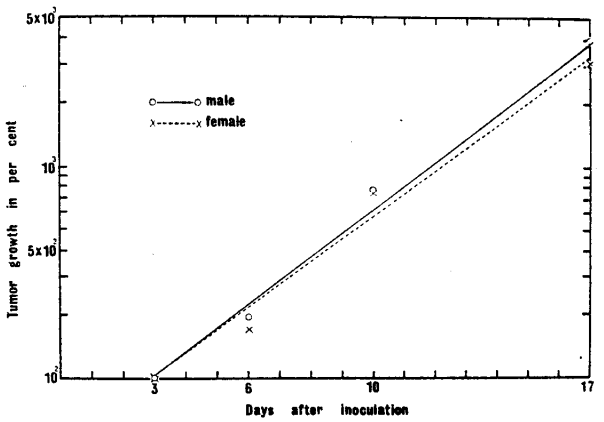

Fig. 4 Rate of tumor growth after subcutaneous inoculation with 2.5×10^6 C3H mouse mammary tumor cells

and intestine cornin from small intestine devoid of mucous membrane of mongrel dog. Cornins were dissolved in physiological saline solution (pH 6.8) as to contain 10, 20 and 30 mg per ml each and were filtered through millipore filter (100 mμ) after centrifugation at 3, 000 r. p. m. for 30 minutes.

According to LITCHFIELD and WILCOXON[19], LD_{50} values of cornins were calculated after intraperitoneal administration of a sufficient number of doses to obtain both 100 and 0 per cent mortality. The LD_{50} value of muscle cornin was 3,250 mg/kg, but the LD_{50} of intestine could not be determined, because there was no death after intraperitoneal administration of cornin, being able to dissolve itself in a limited volume of physiological saline solution.

Procedures

Three series of experiments were carried out with Ehrlich ascites carcinoma.

Series I: The experiments on the antitumor effect of cornin by double-checking methods. (The identical experiments were repeated twice, and the average of the two experiments was given.)

In every experiment, 16 tumor-bearing mice were divided into 4 groups, of which 3 groups were to be treated and the other served as control. The first intraperitoneal or subcutaneous administration of cornin was started from hour 48 of inoculation with tumor cells, which was continued once a day for a week. Each control mouse was injected with 1 ml of physiological saline solution. The body weight of every mouse was recorded and the survival time checked daily for 60 days.

According to the results of preliminary experiments, a standard procedure of antitumor screening was established as shown in Table 2.

Table 2 Standard Procedure of Antitumor Screening for the Ehrlich Ascites Carcinoma

Mouse	Strain ..... ddN strain of mice Sex ..... male Age ..... 6 to 7-week-old Body weight .. 22±2 g Group ..... 4 mice Diet ..... compressed diet (MF) and water
Tumor and inoculation	Ehrlich ascites carcinoma (K-strain), JTC-11 7-day-old 1×10^7cells/mouse, i.p.
Administration	7 times i.p. or s.c. injections starting 48 hours after inoculation

The increase in mean longevity is calculated by the following formula (EGASHIRA *et al.*[4]).

$$L=\frac{lt-lc}{lc}\times100\ (\%)$$

L : longevity increase, lt : longevity of a treated group, lc : that of the control group

The value of L was graded according to the following scheme :

less than 25 %	···	− (no effect)
between 25−50 %	···	± (slight inhibition)
between 50−75 %	···	+ (moderate inhibition)
75 % or more	···	++ (marked inhibition)

Series II: Morphology and number of tumor cells.

Fifteen tumor-bearing mice were divided into 3 groups. The first group was treated with cornin, the second group served as control and the third group used only to determine the number of tumor cells at the beginning of treatment. The following procedures were taken at 48, 96 and 144 hours respectively after the first administration of cornin, which was started 48 hours after inoculation of tumor cells. Tumor ascites of every mouse was taken with a 0.5 ml syringe. A drop of it was used for making preparation, and the remaining part of ascites (0.1 ml) was diluted with Crystal Violet solution for counting the number of tumor cells per ml. The smear was stained with May-Giemsa solution, and the number of tumor cells was counted in a Buerker hemocytometer[13].

Series III: The experiments on the effect of intestine cornin on the Ehrlich ascites carcinoma cells *in vitro*.

Simplified replicate tissue culture was used. The JTC-11 (K-strain) cells used in the present experiments were in the 406th generation *in vitro*. The media employed were 20 % BS-YLE medium (bovine serum 20 %, yeast extract 0.08 %, lactalbumin hydrolysate 0.4 % and Earl's balanced saline solution containing 100 unit/ml of penicillin) and 20 % BS-YLE media containing intestine cornin in the final concentrations of 1.0, 0.5 and 0.1 % respectively. The initial pH of each medium was checked by pH meter and was sterilized through Seitz filter. The initial pH was 7.4.

Seventeen test tubes with tumor cell suspension (1.5 ml) were prepared, and placed horizontally, at an angle of 5°, in an incubator with constant temperature of 37°C. After incubating for 48 hours, 5 test tubes were selected at random, and tumor cells of each were counted. The remaining test tubes were divided into 4 groups of 3 tubes each, the medium containing cornin of respective concentration was added to each of the three cell suspension groups, and standard medium was renewed in the fourth, control group. After incubating for 96 hours, the tumor cells of each group were counted and average number of 3 tubes was recorded. Simultaneously, morphological changes of tumor cells were examined.

Tumor inhibition tests of cornin with C3H mouse mammary tumor were

carried out in the present experiments.

Thirty-two tumor-bearing mice, 16 males and 16 females, were divided into groups of 4 pairs each. Various doses, ranging 10, 20 and 30 mg per ml, of cornin were given to each of 3-pair groups once a day by intraperitoneal or subcutaneous route from 72 to 96 hours after inoculation of tumor cells and continued for 7 consecutive days. To the other pair, control group, was given physiological saline solution in an equal volume.

The approximate sizes of the two maximum perpendicular axes of each tumor were measured by vernier calipers. The mice were sacrificed at the end of the second week after the first administration of cornin, and the tumor removed and weighed. The body weight of the mice was marked and recorded every day during the period of experiment.

Table 3 shows the standard procedure of screening for the C3H mouse mammary tumor.

Table 3 Standard Procedure of Antitumor Screening for the C3H Mouse Mammary Tumor

Mouse	Strain ..... Zb strain of mice Sex ..... male and female Age ..... 8 to 10-week-old Body weight ... 23±2 g (male), 20±3 g (female) Group ..... 4 mice Diet ..... mixed diet and water
Tumor and inoculation	C3H mouse mammary tumor (A-strain) 21-day-old 2.5 to 5.0×10^6 cells/mouse, s.c.
Administration	7 times i.p. or s.c. injections starting 72 to 96 hours after inoculation

The tumor weight ratio was calculated by the following formula (EGASHIRA *et al*[4].).

$$W=\frac{wt}{wc}\times 100\ (\%)$$

W: tumor weight ratio, wt: average of tumor weight of a treated group, wc: that of the control group

The value of W was graded according to the following scheme:

more than 75 % ··· − (no effect)
between 75−50 % ··· ± (slight inhibition)
between 50−25 % ··· + (moderate inhibition)
less than 25 % ··· ++ (marked inhibition)

RERULTS

Series I: Effect of cornin on the Ehrlich ascites carcinoma by double-checking methods.

According to the standard procedure of screening, four various experiments were performed in this series.

Experiment 1 (Fig. 5, Table 4) *and Experiment 2* (Fig. 6, Table 5): Effect of muscle cornin on the Ehrlich ascites carcinoma.

The results of experiments on antitumor effect of muscle cornin by intraperitoneal or subcutaneous administration are summarized in Figs. 5 and 6, and in Tables 4 and 5.

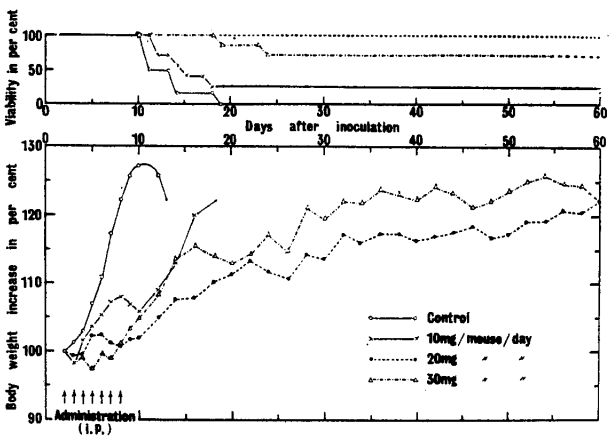

Fig. 5 Effect of muscle cornin by intraperitoneal administration on the Ehrlich ascites carcinoma.

Table 4 Effect of Muscle Cornin by Intraperitoneal Administration

Dose (/M/D)*	Death from disease & toxicity	Viability	Mean longevity (Days) after 30 days,	after 60 days	Longevity increase effect after 30 days,	after 60 days
Control	2/8	0/6	13.3	13.3	.....	.....
10 mg × 7	1/8	2/7	18.7	27.3	± (41%)	++ (105%)
20 mg × 7	1/8	7/7	30.0	60.0	++ (126%)	++ (351%)
30 mg × 7	2/8	4/6	27.1	47.2	++ (104%)	++ (250%)

* /M/D=/mouse/day

Daily doses of 10 mg/mouse had only a slight antitumor effect when administered intraperitoneally, because less than 30 % of the mice regressed from ascites tumor and survived over 60 days, but a large number of the mice died

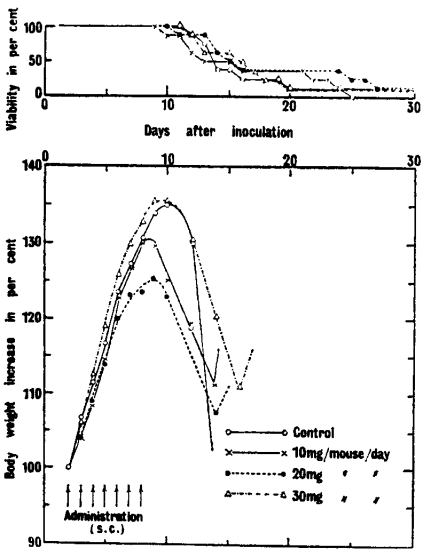

Fig. 6 Effect of muscle cornin by subcutaneous administration on the Ehrlich ascites carcinoma.

just as control. A significant antitumor effect was observed in daily intraperitoneal administrations with doses of 20 mg/mouse. Every mouse failed to develop ascites and the mean body weight curve of the mice was almost equal to the growth curve of normal mice of the same age. Moreover, all these mice survived over 60days, whilst no antitumor effect was observable with those injected subcutaneously.

Seven daily intraperitoneal administrations of 30 mg/mouse also had a marked antitumor effect, but the body weight tended to decrease and 25% of the mice died of acute toxicity of muscle cornin during the period of 7 successive administrations.

Table 5 Effect of Muscle Cornin by Subcutaneous Administration

Dose (/M/D)	Death from disease & toxicity	Viability	Mean longevity (Days) after 30 days	Longevity increase effect after 30 days
Control	0/8	0/8	16.0	.....
10 mg × 7	0/8	1/8	17.4	− (9%)
20 mg × 7	0/8	1/8	19.0	− (19%)
30 mg × 7	0/8	1/8	17.4	− (9%)

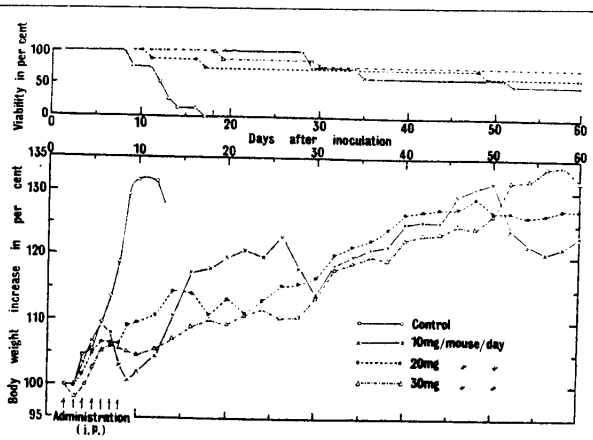

Fig. 7 Effect of intestine cornin by intraperitoneal administration on the Ehrlich ascites carcinoma.

Table 6 Effect of Intestine Cornin by Intraperitoneal Administration

Dose (/M/D)	Death from disease & toxicity	Viability	Mean longevity (Days) after 30 days,	after 60 days	Longevity increase effect after 30 days,	after 60 days
Control	0/8	0/8	12.4	12.4	.....	.....
10 mg × 7	0/8	4/8	29.8	48.0	++ (140%)	++ (287%)
20 mg × 7	0/8	5/8	26.0	47.1	++ (110%)	++ (280%)
30 mg × 7	0/8	6/8	28.6	51.1	++ (131%)	++ (312%)

Subcutaneous administrations had shown no antitumor effect irrespective of doses given.

Experiment 3 (Fig. 7, Table 6) *and Experiment 4* (Fig. 8, Table 7): Effect of intestine cornin on the Ehrlich ascites carcinoma.

The results of experiments with intestine cornin are shown in Figs. 7 and 8, and in Tables 6 and 7.

These results indicate that daily intrapritoneal administrations of intestine cornin in dose of 10 mg/mouse prolongs the survival time as compared with control, though 50 % of the mice died during 60 days' observation. It is interesting to note that daily intraperitoneal administrations appear to be more effective than with the same doses of muscle cornin (Fig. 5 and Table 4).

There could be detected almost no antitumor effect when administered

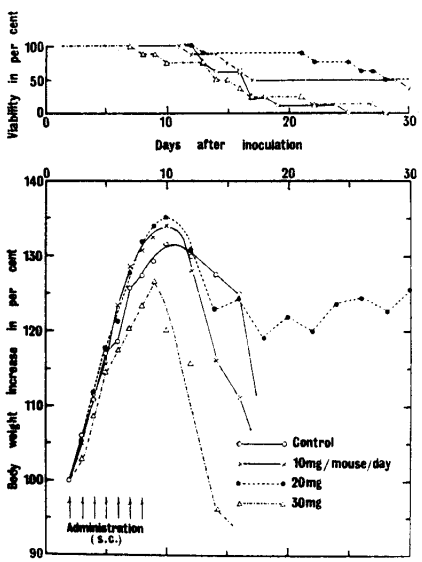

Fig. 8 Effect of intestine cornin by subcutaneous administration on the Ehrlich ascites carcinoma.

Table 7 Effect of Intestine Cornin by Subcutaneous Administration

Dose (/M/D)	Death from disease & toxicity	Viability	Mean longevity (Days) after 30 days	Longevity increase effect after 30 days
Control	0/8	0/8	16.9	.....
10 mg × 7	1/8	2/7	21.3	± (26%)
20 mg × 7	0/8	4/8	26.1	+ (54%)
30 mg × 7	0/8	0/8	16.1	− (− 5%)

subcutaneously.

Daily intraperitoneal administrations of 20 mg/mouse of intestine cornin had a marked antitumor effect just as with muscle cornin, *i. e.* the survival time of the treated mice was significantly prolonged beyond that of control group and the body weight increase by development of ascites was not observed in a large number of mice, which survived over 60 days. On the other hand, 50% of the mice treated subcutaneously with the same doses died of development of ascites tumor during 30 days' observation, but the rest began to fail to develop ascites tumor gradually from about 10 days after inoculation, and seemed to regress from ascites tumor, but finally a large number of the mice died during 60 days after inoculation.

These results shows that the intraperitoneal administrations of 30 mg/mouse had a most remarkable antitumor effect among three various doses of intestine cornin. Almost all the mice so treated regressed completely and remained in good health during 60 days' observation, except the two that died of ascites tumor at the 19th and 29th days after inoculation of tumor cells. During 7 consecutive days of administration of 30 mg/mouse of intestine cornin, there was neither loss of body weight not acute toxicity. No antitumor effect on the Ehrlich ascites carcinoma was shown by subcutaneous administrations of 30 mg/mouse of intestine cornin. The treated mice died earlier than control, and the body weight was seen to decrease rapidly from about the 10th day after inoculation of tumor cells.

Series II: Effect of cornin on the number and the morphology of tumor cells.

Fig. 9 depicts changes in the number of tumor cells at various interval after

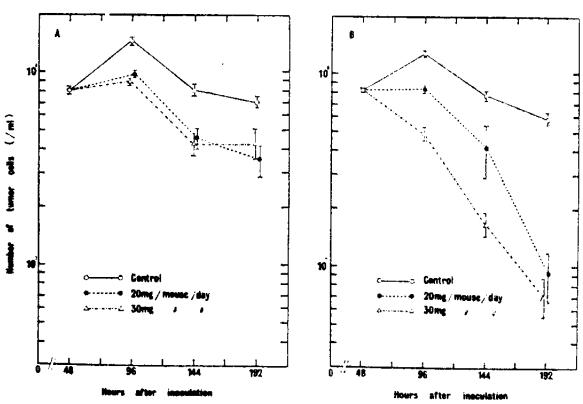

Fig. 9 Effect of cornin on the Ehrlich ascites carcinoma cells.
A : muscle cornin, B : intestine cornin

intraperitoneal administrations of two kinds of cornins. In the control group the maximum number of tumor cells was observed at the 96th hour after tumor inoculation, and afterwards the number of tumor cells was decreased with time.

The cell number of the group treated with daily doses of 20 mg of intestine cornin was almost equal to that of the group treated with daily doses of 20 mg and 30 mg of muscle cornin at the 96th and 144th hours after tumor inoculation, but at the 192th hour it was significantly decreased in the former as compared with that in the latter.

On the other hand, in the group treated with daily doses of 30 mg of intestine cornin the number of tumor cells was apparantly decreased from the beginning of treatment.

Relative tumor cell number in unit volume of ascites is summarized in Table 8.

Table 8 Comparison of the Tumor Cell Number in Unit Volume of Ascites when Treated with Two Kinds of Cornins

Dose (/M/D) / Hours after inoculation	Control	20 mg		30 mg	
		Muscle cornin (rabbit)	Intestine cornin (dog)	Muscle cornin (rabbit)	Intestine cornin (dog)
96	100 %	65.7 %	65.1 %	60.7 %	37.7 %
144	100 〃	55.7 〃	53.5 〃	52.5 〃	21.4 〃
192	100 〃	49.3 〃	16.1 〃	60.1 〃	12.1 〃

Series III: Effect of intestine cornin on the Ehrlich ascites carcinoma cells *in vitro*.

The experimental results are summarized in Fig. 10 and in Table 9.

The proliferation of the tumor cells in the 20 % BS-YLE medium containing respectively different concentrations, *i. e.* 0.1, 0.5 and 1.0 % of intestine cornin, is shown by line numbers, 2, 3 and 4. The line number 1 shows the growth of the cells of the control.

It is obvious from this that the tumor inhibitory effect parallels the concentrations of intestine cornin.

Table 9 Growth Rate of JTC-11 Cells in the Medium Containing Respectively Different Concentrations of Intestine Cornin *in vitro*

Line No.	Final concentration of intestine cornin (%)	Growth rate (%)
1	0	100.0
2	0.1	81.7
3	0.5	57.7
4	1.0	41.0

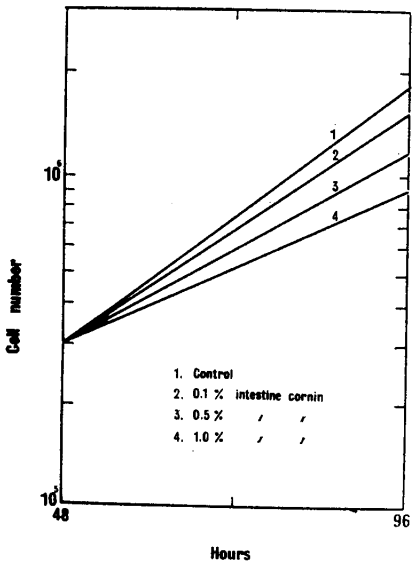

Fig. 10 Effect of intestine cornin on the JTC-11 cells *in vitro*.

Effect of cornin on the C3H mouse mammary tumor

Four various experiments were performed in this series by the standard procedure of screening.

Experiment 1: Effect of cornin by intraperitoneal administration in male.

Table 10 summarizes the results of experiment in male on the antitumor effect of two kinds of cornins, crude muscle cornin and crude intestine cornin, by intraperitoneal administration.

Daily intraperitoneal administrations of muscle cornin in doses of 10 mg/mouse and 20 mg/mouse had no effect on the tumors, and had no influence on the body weight of the tumor-bearing mice. Daily

Table 10 Effect of Cornin on the C3H Mouse Mammary Tumor (male, i. p. administration)

Dose (/M/D)	Toxicity	No. of tumor weighed	Body weight (%) 0	1W.	2W.	(Inhibitory effect / decrease in weight of tumor)
(1) Muscle cornin						
Control	0/4	4	100	102	106	(100 %)
10 mg × 7	0/4	4	100	99	106	− (82 %)
20 mg × 7	0/4	4	100	99	102	− (75 %)
30 mg × 7	2/4	2	100	95	98	± (57 %)
(2) Intestine cornin						
Control	0/4	4	100	103	109	(100 %)
10 mg × 7	0/4	4	100	104	109	+ (48 %)
20 mg × 7	0/4	4	100	101	106	+ (34 %)

30 mg/mouse had only a slight inhibitory effect, but produced intoxication in the treated mice, as manifested by an increasing body weight loss and the mortality of 50%of the treated mice during the period of administrations.

On the other hand, it is of interest that even the daily intraperitoneal administrations of 10 mg/mouse of intestine was more effective on the growth of solid tumors than that of 30 mg/mouse of muscle cornin. The mean tumor weight of the treated mice was less than 50 % of that of the control.

Daily 20 mg/mouse of intestine cornin was most effective in this series of experiments, moreover there was not any toxic effect as manifested by the body weight loss and the mortality of the treated mice.

Experiment 2: Effect of cornin by intraperitoneal administration in female.

By the same techniques described above, the inhibition test was also performed with female mice. The results of this experiment are summarized in Table 11.

Table 11 Effect of Cornin on the C3H Mouse Mammary Tumor (female, i. p. administration)

Dose (/M/D)	Toxicity	No. of tumor weighed	Body weight (%) 0	1W.	2W.	(Inhibitory effect / decrease in weight of tumor)
(1) Muscle cornin						
Control	0/4	4	100	98	99	(100 %)
10 mg × 7	0/4	4	100	101	106	− (79 %)
20 mg × 7	0/4	4	100	102	103	± (74 %)
30 mg × 7	2/4	2	100	105	107	+ (46 %)
(2) Intestine cornin						
Control	0/4	4	100	111	116	(100 %)
10 mg × 7	0/4	4	100	115	128	− (96 %)
20 mg × 7	0/4	4	100	108	124	± (70 %)

Daily intraperitoneal administrations with doses of muscle cornin were somewhat more effective in female than in male. In intraperitoneal administrations of muscle cornin of 30 mg/mouse/day, 50 % of the treated mice died of acute toxicity just as male during the period of administrations, but there was no body weight loss.

Intraperitoneal administration of 10 mg/mouse/day of intestine cornin was effective in male, but not so in female and even the dose of 20 mg/mouse/day had merely a slight inhibitory effect.

Experiment 3: Effect of cornin by subcutaneous administration in male.

The results are given in Table 12.

In the experiment performed by using male animals, the mean tumor weight of treated mice, given 10 mg/mouse/day of muscle cornin, was rather heavier than that of the control at 2 weeks after first administration. There was not any relation between the doses and the inhibitory effect of muscle cornin.

Daily subcutaneous administrations of intestine cornin had a slight inhibitory effect on the growth of tumor, but was not so effective as when administered intraperitoneally. Neither one of cornins showed toxic effect.

Table 12 Effect of Cornin on the C3H Mouse Mammary Tumor (male, s.c. administration)

Dose (/M/D)	Toxicity	No. of tumor weighed	Body weight (%) 0	1W.	2W.	Inhibitory effect (decrease in weight of tumor)
(1) Muscle cornin						
Control	0/4	4	100	101	102	(100 %)
10 mg × 7	0/4	4	100	106	103	− (117 %)
20 mg × 7	0/4	4	100	94	105	± (65 %)
30 mg × 7	0/4	4	100	104	105	− (133 %)
(2) Intestine cornin						
Control	0/4	4	100	102	102	(100 %)
10 mg × 7	0/4	4	100	103	105	± (67 %)
20 mg × 7	0/4	4	100	103	99	± (65 %)

Experiment 4: Effect of cornin by subcutaneous administration in female (Table 13).

Table 13 Effect of Cornin on the C3H Mouse Mammary Tumor (female, s.c. administration)

Dose (/M/D)	Toxicity	No. of tumor weighed	Body weight (%) 0.	1W.	2W.	Inhibitory effect (decrease in weight of tumor)
(1) Muscle cornin						
Control	0/4	4	100	106	113	(100 %)
10 mg × 7	0/4	4	100	108	113	± (70 %)
20 mg × 7	0/4	4	100	107	110	± (57 %)
30 mg × 7	0/4	4	100	117	121	+ (45 %)
(2) Intestine cornin						
Control	0/4	4	100	102	102	(100 %)
10 mg × 7	0/4	4	100	106	108	− (115 %)
20 mg × 7	0/4	4	100	103	105	± (69 %)

In the experiment using female animals subcutaneous administrations of muscle cornin had a slight or moderate inhibitory effect on the tumor, but intestine cornin had hardly any effect when administered subcutaneously.

DISCUSSION

These experiments were planned to examine the effect of cornin not only *in vivo* but also *in vitro*, by using transplantable malignant tumors in mice such as Ehrlich ascites carcinoma and C3H mouse mammary tumor.

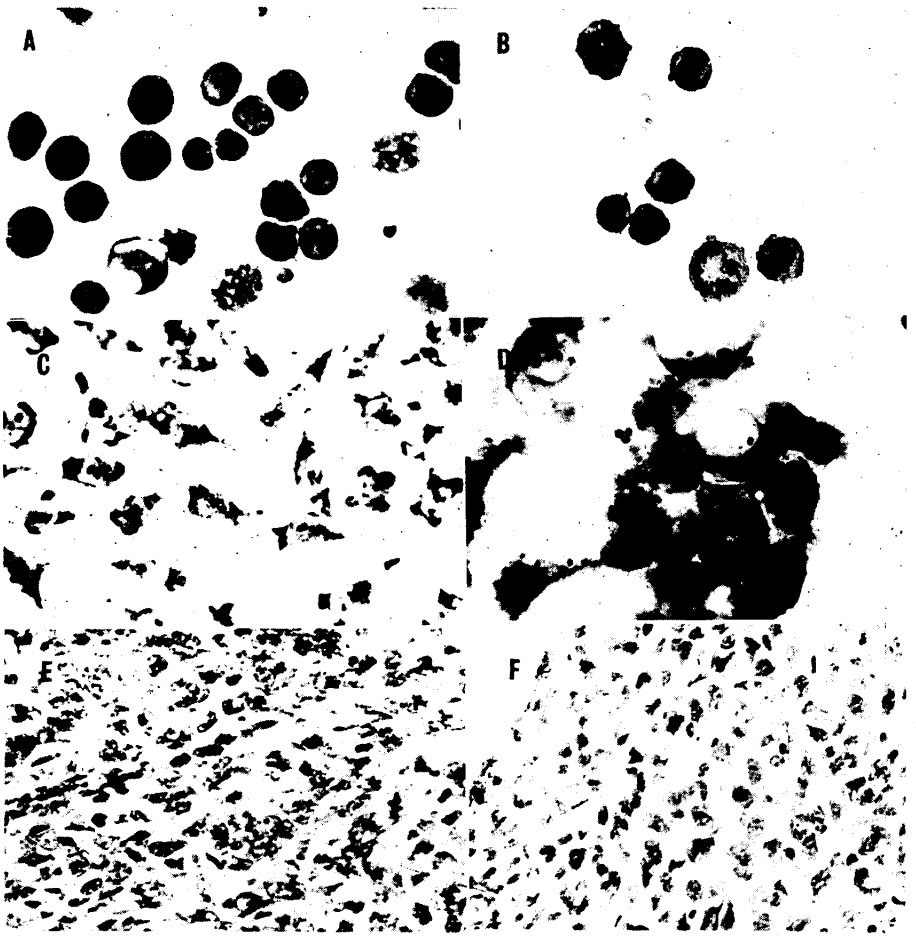

Photo A A general view of the smear of untreated Ehrlich ascites carcinoma cells. (May-Giemsa stain. × 400)

Photo B Ehrlich ascites carcinoma cells after treatment with intestine cornin (30mg/mouse/day for 4 days). The tumor cells do not undergo any morphological changes. (May-Giemsa stain. × 400)

Photo C JTC-11 (K-strain) cells cultured in 20% BS-YLE medium for 96 hours. (May-Giemsa stain. × 400)

Photo D JTC-11 (K-strain) cells grown in the medium containing 1% intestine cornin for 48 hours. They are all degenerated. (May-Giemsa stain. ×400)

Photo E Histological picture of solid C3H mouse mammary tumor. (Hematoxylin and eosin stain. ×400)

Photo F Solid C3H mouse mammary tumor after treatment with intestine cornin (daily doses of 30mg/mouse for 7 consecutive days). No morphological changes at all occur. (Hematoxylin and eosin stain. × 400)

As a result, it was confirmed that both muscle cornin and intestine cornin had a significant inhibitory effect *in vivo*, especially on the Ehrlich ascites carcinoma when administered intraperitoneally, and intestine cornin was more effective than muscle cornin. Further, it was demonstrated that the proliferation of the Ehrlich ascites carcinoma cells was inhibited *in vitro* and morphological change of tumor cells occurred during incubation on addition of intestine cornin. Moreover, as described in a previous paper[28], intestine cornin has a marked inhibitory effect on fibrosarcoma cells induced by DNA of SV40.

On normal cell division, muscle cornin was by far more effective on the cell division of sea urchin eggs (final effective concentration is 10^{-8}g/ml) than intestine cornin (final effective concentration is 10^{-5}g/ml), and the former also had an inhibitory effect on the cell division of regenerating rat liver[18]. But, intestine cornin gave neither antimitotic nor degenerating effects on diploid fibrocytes of normal hamster and human embryonic cells *in vitro*[28].

Summarizing results, it seems that intestine cornin is more effective on the inhibition of malignant cell division than muscle cornin, but in contrast, muscle cornin is rather more effective on the inhibition of normal cell division than intestine cornin.

The administration of large doses of muscle cornin induces bristles, crouches and cramps in the hind legs of mouse for about half an hour, and sometimes brings about death by acute toxicity, but intestine cornin does hardly elicits these symptoms.

The foregoing findings suggest that regardless the same methods employed in the extraction of muscle and intestine cornins, they appear to differ considerably in their biological properties.

Very similar results on not only inhibiting but also stimulating substances from living tissue have been reported by DRUCKREY *et al*[3]. and ROHDENBURG and NAGY[31,32].

According to DRUCKREY *et al.* the tumor inhibitory activity differed appreciably various tissues; homogenates obtained from spleen and lung of rats had a marked inhibitory effect, while those from other organs were less effective. And, ROHDENBURG and NAGY described that by using the culture of protozoan, *Colpidium campylum*, two substances; one, stimulating agent and the other, inhibiting agent, were at first found in human urine, and later cell division stimulating and inhibiting substances were isolated from various tissue, *i. e.* from normal kidney, liver and spleen of human and rabbit, etc, and from benign and malignant tumors. The substances extracted from kidney, liver and malignant tumors had especially a significant stimulating activity on the cell division.

In addition, there is an interesting result to show the difference in species of animals (HERBUT and KRAEMER[9]): *e. g.* a result of test on the inhibitory

activity of physiological saline extracts of livers from several species of animals has revealed that only extract of guinea pig liver has a very significant tumor inhibitory activity *in vitro*, but the liver extracts from other animals are not so markedly effective.

There are many reports on tumor inhibiting and/or promoting substances in liver, but these substances differ from each other in their chemical and physical properties.

BRUES *et at*[2]. reported that saline and alcoholic extracts of adult liver, *i. e.* rat, mouse, fowl, etc, inhibited the growth of normal fibroblasts from chick embryo heart and also mouse sarcoma 180. They indicated that there are at least two inhibitory constituents of liver, and one of these inhibitors is ethanolamine.

NAKAHARA and FUKUOKA[21] demonstrated that aqueous extract of normal liver has a potent carcinostatic factor, which is not contained any other tissues or liver of tumor-bearing animals, and it was later reported by HOZUMI *et al*[11]. that the activity of this liver factor is increased by the successive procedure of alcoholic extracts. The results of isolation indicated that these liver fractions consist of simple sugar or small oligosaccharides and polysaccharides[45]. In spite of a strong inhibitory effect on the growth of the Ehrlich ascites carcinoma cells *in vitro*, these active fractions have not only 'and inhibitory effect *in vitro*, but also the whole liver extract has the growth-promoting effect on the tumors[22].

KATSUTA *et al*[16,17]. found chick embryo extract and liver extract of normal rats to possess an inhibitory effect on the proliferation of rat ascites hepatoma cells *in vitro*, and later, SUZUKI[36] confirmed that liver of normal rats exhibits such an inhibitory effect.

Now, there are also many studies on the growth-inhibiting substances from various kinds of living tissues except liver by various methods.

In 1937, SIMMS and STILLMAN[34,35] found that digestion of fresh adult tissue with trypsin stimulated its initial growth *in vitro* sooner and more rapidly, because of the removal of an inhibitory material from tissue due to the proteolytic action of trypsin. They isolated a growth inhibitor against the growth of normal adult chicken fibroblasts from chicken, dog and sheep aortas by mild tryptic digestion and they considered that the growth inhibitor corresponded to lectein in its physical and chemical properties.

Similar works have been carried out chiefly in tissue culture by PARSHLEY [29,30]. She demonstrated that tissue extracts have inhibitory and promoting effects not only on various malignant cells, but also on normal fibroblasts. Namely, the growth of normal connective tissue cells and the cells of 25 human tumors were inhibited by 75～100 %, and cells from tumors of mesenchymal origin and fibroblasts in other types of tumors were affected most, but in many in-

stances, normal fibroblasts from adult chicken aorta were less affected. PARSHLEY has isolated those extracts that have variable amounts of inhibitory and/or promoting effects *in vitro*, from normal connective tissue constituents (muscle, tendon, aorta, etc.) by methods of mild tryptic digestion, and suggests that inhibitors might be a mucopolysaccharide-nucleic acid-protein complex.

In the serial works SZENT-GYÖRGYI[37,38,42,43] and SZENT-GYÖRGYI *et al*[5,6,7,39,40,41]. succeeded in fractionation of the biological active substances from thymus gland and in isolation of two fractions, one of which had a strong inhibitory effect on various malignant growth, while the other had a strong promoting effect on the same cells. The former was called "retine", and the latter termed "promine". Later, the growth-promoting substance and another with sterilizing activity were separated from these substances, the latter was termed "infertine". These substances, especially retine, were extracted from thymus gland, several other tissues (*i. e.* tendon, blood vessel and muscle), molluscus and human urine. Retine is thought to be a methylglyoxal derivative. They are of the opinion that retine may represent a universal cell constituent, and that retine and promine may actually be the regulators of cell division. PARSHLEY also gave her opinion very similar to SZENT-GYÖRGYI's, that is, a balance of stimulatory and inhibitory substances is a controlling factor of normal growth.

Tissue extracts with promoting effect have not been found in our experiments, but it seems that there are two different kinds of substances; one with a greater inhibitory activity on the normal cell division and the other with a more marked inhibitory activity on the malignant cell division as viewed from our serial experiments with cornin. In comparing retine with cornin, they differ in their chemical and physical properties, and likewise in their absorption peak.

There are several reports on the growth-inhibiting substances from animals except vertebrates. Cleavage retarding factors were found from sea urchin eggs, ovaries, testes and gut by WOLFSON[46], and from starfish ovaries by HEILBRUNN *et al*[8]. Growth-inhibiting agent was extracted from Marcenaria by SCHMEER[33].

According to recent studies on histones, it is proved that histones have an inhibitory effect on the growth of tumor cells. BLAZSEK and GYERGYAY[1] stated that the histone either from fresh calf thymus gland or the Ehrlich ascites carcinoma cells had a strong inhibition of the growth of the Ehrlich ascites carcinoma *in vitro*, while that the immediate administration of histones after inoculation of the Ehrlich ascites carcinoma cells or treatment of animal with both histones did not result in any inhibitory effect on tumor growth.

As stated above, many growth-inhibiting substances have been from various sources by different methods, indicating that such a variety of inhibitors do exist in living tissues. Cornin may be one of the inhibitory substances in living tissues, and needless to say, it is necessary to carry out further studies on the purifica-

tion of cornin and to examine deeper into its biological, chemical and physical properties.

SUMMARY

1. In the present experiments, Ehrlich ascites carcinoma (K-tsrain), JTC-11, and C3H mouse mammary tumor (A-strain) were used to study the inhibitory effects of two kinds of cornins, crude muscle cornin and crude intestine cornin.

2. Daily intraperitoneal administrations of both cornins had shown a marked inhibitory effect on the Ehrlich ascites carcinoma.

3. Intestine cornin was more effective on the inhibition of the growth of the Ehrlich ascites carcinoma than muscle cornin when administered intraperitoneally.

4. Daily subcutaneous adminstrations of muscle cornin had no effect, but doses of 10 mg/mouse/day or 20 mg/mouse/day of intestine cornin had a slight or moderate inhibitory effect on the Ehrlich ascites carcinoma.

5. Intestine cornin had an inhibitory effect on the growth of JTC-11 cells *in vitro*, and made the tumor cells to undergo morphological changes during incubation.

6. Daily intraperitoneal administrations of muscle cornin had hardly any effect on the C3H mouse mammary tumor, but intestine cornin was evidently effective in male.

7. Intraperitoneal administrations of intestine cornin proved to be hardly effective on the C3H mouse mammary tumor, but only in the dose of 30 mg/mouse/day, it had a moderate inhibitory effect in female.

8. Daily subcutaneous administrations of muscle cornin had no effect on the C3H mouse mammary tumor, but intestine cornin had a slight effect in male.

9. Muscle cornin had a slight or moderate effect on the C3H mouse mammary tumor, but intestine cornin was hardly effective in female when administered subcutaneously.

10. Repeated intraperitoneal administrations in doses of 30 mg/mouse/day of muscle cornin produced intoxication in the treated mice.

11. In general, it seems that intestine cornin is more effective on the inhibition of tumor growth than muscle cornin.

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