STUDIES ON ANTI-TUMOR ACTIVITY OF SOEDOMYCIN, A NEW ANTI-TUMOR AGENT

I. Inactivation effect on CAE and anti-tumor effect on Ehrlich ascites tumor and Sarcoma 180 of mice.

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新抗腫瘍物質 Soedomycin の抗腫瘍性に関する研究（第1報）

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1965年添田は Streptomyces hachijoensis (Hooya et al, 1951) の培養濾液から抗腫瘍物質を分し Soedomycin（別名 M3 物質とも云う）と命名した．本物質の抗腫瘍性を Ehrlich 腹水癌並に Sarcoma 180に対して検討を加えた．

従来の抗癌性物質の殆んどが腫瘍細胞の重要な細胞成分の合成を阻害し或は之に相制作用を及ぼすことに依り細胞分裂の阻止，細胞機能の阻害，細胞の崩壊等を起し之に基いて抗腫瘍性を発揮すると考えられているのに対し，本物質は著者の発見した癌原子（carcinogenic agent）に直接作用し之を不活化することに依り抗腫瘍性を発揮することを認めた，また本剤には白血球減少，貧血，肝を始めとする臓器障害等の中毒反応が認められないのも本剤が他の抗癌物質と異り抗細胞性物質でない為と著者は考えている．

Synopsis

In 1965, Soeda had isolated an anti-tumor substance from agitation culture broth of Streptomyces hachijoensis n. sp. (Hosoya et al, 1951) and named Soedomycin. Its anti-tumor activity against animal tumors has been studied with Ehrlich ascites tumor (EAC) and Sarcoma 180 of mice, adopting both ascites cells as target cells.

Anti-tumor activity of Soedomycin cannot be regarded as of anti-cellular, because it does not seem to exert its anti-tumor activity by interaction with vital cellular constituents of tumor or by interferring with synthesis of vital cell components responsible for cell reproduction.

Here I report on experimental evidences which may support such view, and some aspects of the mechanism by which this agent may exert its anti-tumor activity are discussed.

Introduction

In 1961, Soeda and Sumiyama reported on the experimental studies, in which some specific agent responsible for induction of ascites tumor in mice was shown to be present in the supernatant of ascites fluid of mice with EAC, which was entirely made cell-free by centrifugation at 20,000 r.p.m. for 30 minutes. This cellfree supernatant proved capable of inducing ascites tumor in mice.

Similar studies were carried out on Yoshida sarcoma of rats and Sarcoma 180 of mice and it was shown

that this was also true even in the cases of such tumors. I named such agent of Ehrlich ascites tumor CAE, which means "carcinogenic agent of Ehrlich ascites tumor" (Soeda, 1961).

Recently we have tested on prophylactic and therapeutic effect of a modified preparation of CAE-containing cell-free supernatant and found that it can act as a vaccine for protection of mice against ascites tumor after i.p. implantation of tumor cells in a dose fatal for normal mice.

This modified preparation of cell-free ascites was named EAD, which means Ehrlich agent derived from Ehrlich asictes cells (Soeda, 1964).

In 1951, Hosoya et al had isolated an antibiotic substance, named Trichomycin, from mycelia of Streptomyces hachijoensis. Another antibiotic substance was recently isolated from agitation culture broth of the same strain of Streptomyces and named Soedomycin (Soeda, 1965). This agent was also called by the name of M 3 substance, because it was the third anti-tumor agent found in our laboratory. Now it is obtained in an amorphous, white and powdery form and its i.p. LD_{50} for mice is about 120 mg/Kg. Its chronic toxity has not been recognized. When given i.v. into rabbits or i.p. into mice, it does not cause leukopenia, but rather slightly increases the white cell count some hours after administration. Daily injection of this agent for as long as 3 months did not induce leukopenia in animals. It has neither antibacterial nor antifungal activity as shown in Table 1.

Table 1. In vitro activities of M 3 against representative bacterial and fungal strains

Organisms	Concentration of inhibition (mcg)
1. Staphylococcus aureus 209-P	More than 300
2. Micrococcus flavus	More than 300
3. Escherichia coli communis	More than 300
4. B. subtilis NRRL B-558	More than 300
5. Baccillus agri	More than 300
6. Ps. aeruginosa Tsuchijima	More than 300
7. Mycobacterium phlei Thimoty	More than 300
8. Candida albicans	More than 300
9. Aspergillus niger	More than 300

More recently a series of experiments have been carried out for quantitative analysis of anti-tumor activity of M 3 substance against ascites cells of both Ehrlich ascites tumor and Sarcoma 180 of mice. Graded doses of M 3 were mixed with cell-free ascites fluid or tumor cell suspension, and the mixtures were kept at 37°C for an hour and inoculated i.p. into mice immediately thereafter. By this means the minimum dose for inactivation of 10^5 or 10^6 tumor cells was grossly estimated.

Therapeutic effect of M 3 substance on tumor growth in mice was also examined and in some experiments another anti-tumor agent called Marimycin (M-2 substance) was used in combination with M 3 to test their synergetic activity against tumor cells.

The purpose of this report is to describe the experimental results and to discuss the mechanism of anti-tumor activity exerted by M 3 substance.

Materials and Methods

Animals: Male mice of DDS strain weighing about 20 gm were used for studies.

Tumor cells: Ehrlich ascites tumor cells and Sarcoma 180 cells were adopted as target cells. They

have been maintained by serial transplantation from one group of mice to another at appropriate intervals for more than 5 years.

CAE: Ascites fluid was collected from mice with Ehrlich ascites tumor, and after the cell count was done it was diluted with saline to prepare a dilution containing 10^7 tumor cells per ml. This dilution was centrifuged at 4,000 r.p.m. for 30 minutes to obtain cell-free supernatant. This was used for experiments with CAE after serially diluted with saline.

M 3 substance (Soedomycin, 1965): Fifty per cent solution of zinc chloride was added to filtrate of agitation culture broth of Strptomyces hachijoensis n.sp. (Hosoya et al, 1951) in the proportion of 1:50 to 1:100 and the resultant precipitate was removed by centrifugation at 3,000 r.p.m. for 15 minutes. After this precipitate was dissolved into 10% disodium phosphate solution it was mixed with an equal volume of methanol, and centrifuged at 3,000 r.p.m. for 30 minutes to remove developed precipitate. This was dissolved into distilled water and lyophilized after it was dialyzed against distilled water. By this means M 3 may be obtained in an amorphous, white and powdery form. I have defined, tentatively, aminimum dose of M 3 as 1.0 unit, which is sufficient for inactivation of 10^6 EAC cells within an hour at 37°C, in vitro test devised by myself.

Experimental Results

Exp. 1. Ascites tumor induced in mice by i.p. inoculation with CAE

A dilution of ascites fluid containing 10^7 EAC cells per ml was initially prepared and centrifuged at 4,000 r.p.m. for 30 minutes. Total 4 test mice given i.p. 0.1 ml of this cell free fluid developed ascites

Table 2 Transplantability of EAC by cell-free supernatant fluid

Mice	Supernatant fluid corresponding to	Additional medication	Outcome of test mice
1.	10^6 E C C	／	● (27)
2.	10^6 〃	／	● (28)
3.	10^6 〃	／	● (39)
4.	10^6 〃	／	● (39)
5.	10^6 E A C C	240 mcg M 3	○ (29) ● (45)
6.	10^6 〃	120 〃	○ (29) ○ 140
7.	10^6 〃	60 〃	○ (29) ○ 140
8.	10^6 〃	30 〃	● (39)

* (27) means ascites tumor death on the 27th day; ○ (29) means rechallenge with 10^5 EAC cells; 140 means survival on the 140th day.

tumor and died after 27, 28, 39 and 39 dys respectively (Table 2). On similar experimental results we have already reported in 1959[9], in which ascites fluid centrifuged at 20,000 r.p.m. for 30 minutes was also used with a similar outcome. Anyway it is of much interest that ascites tumor can be transplanted by cell free supernatant, because it has been generally believed that intact tumor cells are essential for transplantation of asictes tumors.

Exp. 2. Inactivating effect of M 3 on CAE in cell free ascites fluid

A dilution of ascites fluid containing 10^7 tumor cells per ml was initially prepared and then centrifuged in like manner to get cell free ascites fluid corresponding to 10^7 tumor cells per ml. M 3 was mixed

with such fluid in graded proportions of 2,400 mcg/ml, 1,200 mcg/ml, 600 mcg/ml and 300 mcg/ml and these mixtures were kept at 37°C for an hour before use. Total 4 mice were given i.p. 0.2 ml of respective mixtures. One mouse given 0.2 ml of the mixture in the proportion of 300 mcg/ml developed ascites tumor and died 39 days thereafter, but the remaining 3 mice survived without any signs of ascites tumor up to the 29th day, when they were challenged with 10^5 intact tumor cells. Two of 3 mice proved completely immune against this cell dose and survived until 140th day, when they were killed and histologically examined. No findings characteristic of tumor were recognized (Table 2).

Exp. 3. Inactivating effect of M 3 on Sarcoma 180 cells

Ascites was collected from mice with Sarcoma 180 and a dilution of ascites fluid containing 10^7 tumor cells per ml was initially prepared with saline and centrifuged at 4,000 r.p.m. for 30 minutes. After the supernatant was discarded, saline was added to the sedimented tumor cells to restore the original volume

Table 3 Inactivating effect of M 3 on Sarcoma 180 cells

Mice	Sar. 180 cells devoid of sup. fluid	Dose of added M 3	Outcome of test mice
1.	10^6	50 mcg	047
2.	10^6	25	● (27)
3.	10^6	25	● (25)
4.	10^6	12.5	● (27)
5.	10^6	6.2	● (22)
6.	10^6	3.1	● (19)
7.	10^6	1.6	● (19)
8.	10^5	0.8	● (25)
9.	10^5	／	● (25)
10.	10^5	／	● (23)
11.	10^5	／	● (23)

* (25) means ascites tumor death on the 25th day; ○47 means survival on the 47 th day without signs of ascites tumor;

Table 4 Inactivating effect of M 3 on Sarcoma 180 cells

Mice	Sar. 180 cells devoid of sup. fluid	Dose of added M-3	Outcome fo test mice
1.	10^5	50 mcg	○47
2.	10^5	50	○47
3.	10^5	25	○47
4.	10^5	25	○47
5.	10^5	12..5	○47
6.	10^5	6.25	○47
7.	10^5	3.13	● (25)
8.	10^5	／	● (23)
9.	10^5	／	● (22)
10.	10^5	／	● (22)

● (22) means ascites tumor death on the 22nd day; ○47 means survival on the 47th day without signs of ascites tumor;

and then to each 1.0 ml of this cell suspension were added graded doses of M 3 substance and the mixtures were kept at 37°C for an hour before use. At least 50 mcg of M 3 was required for complete inactivation of 10^6 washed tumor cells. Less than 25 mcg was not enough for this purpose, because all mice given i.p. 10^6 washed tumor cells pretreated with less than 25 mcg of M 3 developed ascites tumor and died within 27 days (Table 3).

With 10^5 washed tumor cells, about 6 mcg seemed to be sufficient for complete inactivation of them.

Exp. 4. Therapeutic effect of M 3 on mice implanted with Ehrlich ascites cells Test (1) Treatment with a single i.p. injection of 1.28 units of M 3

Mice were given i.p. 10^4 or 10^5 tumor cells and 24 hours later they were treated with a single injection of 1.28 units of M 3. Control mice i.p. inoculated with 10^3 to 10^5 tumor cells received no treatment thereafter.

Table 5 Therapeutic effect of M 3 on mice inoculated with a dose of 10^4 to 10^6 cells of Ehrlich ascites tumor

EAC cell dose	Total dose of M 3	Schedule of administration	Number of mice	Ascites tumor death on	Escaped from EAC
10^4	1.28 u.	A single dose, 24 hrs after cell inocul.	2	1 (28)	1
10^5	1.28 u.	〃 〃	2	2 (20, 30)	0
10^3	〃	〃	2	2 (17, 31)	0
10^4	〃	〃	2	2 (28, 28)	0
10^5	〃	〃	2	2 (13, 29)	0
10^5	200 mcg	daily 50 mcg for 4 days (50X1X4)	4	2 (30, 30)	2
10^5	〃	〃	4	4 (18, 23, 30, 44)	0
10^5	1,200 mcg	200mcg twice a day for 3days(200X2X3)i.p.	3	1 (29)	2
10^5	600 mcg	200mcg once a day for 3days(300X1X3)i.p.	3	2 (33, 49)	0*
10^5	〃	〃	3	3 (20, 25, 30)	0
10^6	400 mcg	100 mcgX2X1 plus 50 mcgX2X2 i.p.	4	3 (15, 15, 35)	1
10^6	200 mcg	50 mcg X2X2	4	3 (13, 20, 25)	1
10^6	〃	〃	4	4 (17, 17, 19, 20)	0

* one mouse developed a solid tumor at the site of cell implantation.

Only one mouse given 10^4 tumor cells and treated with M 3 did escape from ascites tumor death and survived for more than 3 months, but other mice all died within 31 days as a result of ascites tumor(Table 5).

Test (2) Treatment with daily i.p. injection of 50 mcg of M 3

Total 8 mice given i.p. 10^5 tumor cells were equally divided into 2 groups. Mice in the first group were treated with daily i.p. injections of 50 mcg M 3 for 4 successive days, starting 24 hours after tumor cell inoculation, while mice in the second group received no treatment as controls. In the first group, 2 of 4 mice could escape from tumor deaths and survived for more than 3 months, but the remaining 2 mice died as a result of ascites tumor within 18 to 44 days (Table 5).

Test (3) Treatment with daily i.p. injection of 100 mcg of M 3

Total 12 mice were inoculated with 10^6 tumor cells. They were divided into 3 groups. Mice in the first group received 2 injections of 100 mcg M3 on the next day, and they were further treated for the following 2 days with 50 mcg M 3, twice a day. Mice in the second group did not receive M 3 on the next day and they were treated with 50 mcg M 3, twice a day, for the following 2 days. Despite such different schedules of treatment, each 3 mice in both groups developed ascites tumor and died within 15 to 39 days. Each one mouse in both groups could escape from tumor death and survived for more than 3 months (Table 5).

Test (4) Treatment with daily i.p. injection of 200 to 400 mcg of M 3

Total 9 mice were inoculated with 10^5 tumor cells and 24 hours later 3 mice of the first group were treated with i.p. injection of 200 mcg M 3, twice a day, for 3 days, while 3 mice of the second group were treated with i.p. injection of 200 mcg M 3, once a day, for 3 days. The third group served as control. In the first group, 2 of 3 mice did not develop ascites tumor and were killed on the 35th day for histological examination. No findings characteristic of tumor were revealed. In the second group 2 mice developed ascites tumor and died, but one mouse developed a solid tumor at the site of tumor cell inoculation. This mouse was further treated with subcutaneous injection of total 2.2. mg EAD (modified preparation of CAE-containing cell free ascites) during the course of 8 days. The solid tumor gradually decreased in size and eventually disappeared within about 2 months (Table 5).

From these experimental results, i.p. administration of a daily dose of 400 mcg M 3 for 3 days seemed to be grossly required to rescue the majority of mice, which had been i.p. inoculated with 10^5 tumor cells.

Exp. 5. Therapeutic effect of combined use of M 3 and M 2 on mice inoculated with Ehrlich ascites tumor cells

Marinamycin is an anti-tumor agent isolated from agitation cultures of Streptomyces mariensis (Soeda, 1957). It was later chemically purified to remove undesirable side-effect and this purified form was called by the name of M-2.

Test (1) Combined use of M 3 and M 2 posterior to tumor cell implantation

Mice were i.p. inoculated with 10^5- Ehrlich ascites tumor cells, and 3 mice of the first group were treated with a single injection of 240 mcg M 3, while 3 mice of the second group received an additional injection of 230 mcg M-2. In both groups 2 mice did not develop ascites tumor, but another mouse could not escape

Table 6 Therapeutic effect of combined use of M 3 with M 2 on mice inoculated with 10^4 or 10^5 EAC cells

EAC cell dose	Total dose of M 3	Schedule of i.p. administration		Number of mice	Ascites tumor death on	Escaped from EAC
		M 3	M 2			
10^5	240 mcg	240X 1 X 1	/	3	1 (26)	2
10^5	240 mcg	240X 1 X 1	230X 1 X 1	3	1 (30)	2
10^5		/	/	3	3 (18, 28, 31)	0
10^4	400 mcg	50X 2 X 4	/	4	2 (31, 35)	2
10^4	400 mcg	50X 2 X 4	230X 2 X 4	4	0	3*
10^4		/	/	3	3 (21, 24, 26)	0

* one mouse died by accident on the 7th day.

from ascites tumor death (Table 6). Similar test was done with 10^4 tumor cells. Mice were i.p. inoculated with 10^4 tumor cells, and 4 mice of the 1st group were treated with i.p. injection of 50 mcg M 3, twice a day, for 4 days (total 400 mcg), while 4 mice of the 2nd group were treated with i.p. injection of 50 mcg M 3 incombination with 230 mcg M 2, twice a day, for 4 days (total 400 mcg M 3 and 1,840 mcg M 2). Two of 4 mice of the 1st group developed ascites tumor and died within 35 days, but the other 2 could escape from ascites tumor deaths, while 3 mice of the 2nd group survived without clinical signs of ascites tumor for more than 3 months. Another mouse died by accident on the 7th day (Table 6).

Test (2) Combined use of M 3 and M-2 prior to and posterior to tumor cell implantation

Mice were given i.p. 20 mcg M 3 and 24 hours later they received an i,p. implantation of 10^4 tumor cells. Starting on the next day they were treated with i.p. injection of 20 mcg M 3, once a day, for 4 consecutive days (total 100 mcg M 3). Mice of another group were given i.p. 20 mcg M 3 and 1,000 mcg M-2 and on the next day they received an i,p. inoculation of 10^4 tumor cells, and then they were further treated with i.p. injection of a daily dose of 20 mcg M 3 plus 230 mcg M-2 for 4 consecutive days. Six control mice developed ascites tumor and died within 26 to 36 days, but one mouse of the 1st group and 3 mice of the 2nd group did escape from ascites tumor death.

Discussion

A dose of 10^2 cells of Ehrlich ascites tumor may be regarded as sufficient to kill the majority of usual mice of DDS strain. According to our experimental evidences, a cell dose of 10^8 can be regarded as absolutely fatal for usual mice of this strain, except for naturally resistant cases very rarely found among so many mice.

In contrast to this, a dose of CAE corresponding to 10^3 tumor cells may be also regarded as sufficient to kill the majority of usual mice. It should be of much interest that entirely cell-free supernatant of ascites fluid of mice with ascites tumor retains an almost similar transplantability of ascites tumor, because it has been generally believed that intact tumor cells are essential for transplantation of such tumors.

Soedomycin proved active against CAE. About 60 mcg M 3 proved capable of completely inactivating, in vitro, CAE corresponding to 10^6 tumor cells.

For this reason, it seems reasonable to consider that there are at least 2 different groups among so-called anti-tumor agents, namely an anti-cellular group and an anti-CA group. For instance, anti-metabolites such as 6-MP, aminopterin and azaserine may be regarded as anti-cellular drugs, because they exert their anti-tumor effect by interferring with synthesis of cellular constituents which will disturb cell division. It is also presumed that physiological activity of the alkylating agents results from their interaction with vital cellular constituents, which if extensive enough, will disturb cellular function and thus produce toxic manifestations.

In contrast to these agents, M 3 should be classified as a member of the anti-CA group, because it can, in vitro, inactivate CA of Ehrlich ascites tumor which may lead to the complete loss of vital capacity of CA to induce ascites tumor in mice. M 3 must exert its anti-tumor effect by interferring with vital activity of CA responsible for malignation of some host cells. Here I said "Malignation of some host cells", because we cannot seek the origin of ascites tumor cells among other than host cells. It seems likely that CA liberated from tumor cells may retain tumor-inducing capacity even if it being separated from tumor cells and exert its vital activity against some host cells which will cause their transformation to malignant tumor

cells. If CA of Ehrlich ascites tumor is inactivated by anti-tumor effect of M 3, it will become an inert form which is incapable of inducing malignant of host cells.

Anti-tumor effect of M 3 on intact tumor cells of Sarcoma 180 was also tested, and about 50 mcg M 3 proved to be rquired for complete removal of transplantability of 10^6 washed tumor cells in vitro. This phenomenon may be either due to alteration of vital cellular constituents of tumor or to inactivation of all CA present within tumor cells. It is not clear at the present time whether tumor cells will lose viability by such treatment with M-3, however, it seems certain that M 3 may alter them to an inert form as far as transplantability of tumor cells being concerned. In connection with these unknown aspects many problems will remain to be solved in future research.

By far a larger dose of M 3 was required for therapy of mice which had been i.p. inoculated with 10^5 to 10^6 tumor cells, although the experimental results were considerably variable from one test to another resulting from obscure reasons. grossly speaking, i.p. administration of a daily dose of 400 mcg for 3 days may be necessary for rescue of the majority of mice inoculated with 10^5 cells of Ehrlich ascites tumor.

Thus M 3 proved not only capable of inactivating CA of Ehrlich ascites tumor or intact cells of Sarcoma 180, but also capable of exerting beneficial therapeutic effect on mice implanted with Ehrlich tumor cells. The most importat point may be that M 3 should be regarded as a member of the anti-CA group of anti-tumor agents, which will probably contribute to further solution of many problems in regard to the nature of malignancy of tumor cells or the general concept of the mechanism by which anti-tumor agents can exert their inhibitory effect on tumor growth. At least, the beneficial inhibitory effect of M 3 substance on tumor cells does not seem to be due to interaction with vital cellular constituents of tumor, but it seems likely that it directly inactivates vital activity of the carcinogenic agents (CA) of ascites tumors.

Summary and Conclusion

An anti-tumor agent, named Soedomycin, was prepared from agitation culture broth of Streptomyces hachijoensis by Soeda (1965). It has neither anti-bacterial nor anti-fungal activity, but it proved to be active against malignant tumors such as Ehrlich ascites tumor and Sarcoma 180 of mice and Yoshida sarcoma of rats. It was also called by the name of M 3, because it was the third antitumor substance found in our laboratory.

Recently a series of experiments have been performed for quantitative analysis of anti-tumor activity of M 3 against Ehrlich ascites tumor and Sarcoma 180.

1) About 60 mcg M 3 was almot sufficient for inactivation of CA present in 0.1 ml of the supernatant of diluted ascites fluid containing 10^7 cells of Ehrlidh ascites tumor per ml. In contrast to this, at least 50 mcg M 3 was required for complete removal of transplantability of 10^6 washed cells of Sarcoma 180, while only about 6 mcg M 3 seemed enough to inactivate 10^5 cells of the same tumor.

2) Therapeutic activity of M 3 against growth of Ehrlich ascites tumor in mice was tested. Although the results were considerably variable from one test to another, i.p. administration of a daily dose of 400 mcg M 3 for 3 days seemed to be necessary to rescue the majority of mice from ascites tumor deaths, which had been inoculated with 10^5 tumor cells.

3) Synergetic effect of simultaneous use of M 3 and M-2 was tested in like manner, and the best result was obtained from the case in which mice were inoculated with 10^4 EAC cells and treated with i.p. injection of a daily dose of 50 mcg M 3 plus 230 mcg M-2, twice a day, for 4 successive days, starting 24 hours

after cell inoculation. By such schedule of therapy, almost all mice inoculated with 10^4 cells of Ehrlich ascites tumor were rescued from ascites tumor deaths. In general, it seems better to employ a relatively small daily dose of M 3 and to repeat injection for more than 3 days for treatment of animals. Simultaneous injection of M-2 appeared to enhance the anti-tumor activity of M 3, the exact reason of which is not clear at present. Although M-2 has a slight anti-tumor activity, its synergetic effect my be due to its lymphocytotic property which will accelerate immunization of host animals.

Anyway, from these experimental results, it seems reasonable to consider that there are, at least, two groups among anti-tumor agents, and M 3 should be classified as a member of anti-CA group, which exerts its anti-tumor effect by interaction with vital activity of the carcinogenic agent for tumors, and not by interaction with vital cellular constituents of tumors.

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