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PRODUCTION OF METASTASES BY TREATMENT WITH CARCINOSTATIC AGENTS

III. LOW CONCENTRATION OF CARCINOSTATIC AGENTS ON THE CELLS*

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ABSTRACT

The effects of treatment with various doses of carcinostatic agents on tumor growth have been studied.

In animal experiments, treatment with low concentrations of nitrogen mustard N-oxide resulted in an early death of mice bearing Ehrlich ascites carcinoma and an increase in the number of circulating Yoshida sarcoma cells. The growth of subcutaneously implanted Yoshida sarcoma was stimulated after contact of the cells with low concentrations of nitrogen mustard N-oxide *in vitro* or intraperitoneally.

Low concentrations of Mitomycin-C and Chromomycin A_3 also showed increase of tumor growth and metastasis formation.

In vitro study showed that the proliferation of HeLa cells was stimulated by adding low concentrations of nitrogen mustard N-oxide, Mitomycin-C, and Chromomycin A_3 into the culture media.

In clinical studies, much more cancer cells were released into the stomach cavity after the administration of nitrogen mustard N-oxide, Mitomycin-C, and Chromomycin A_3.

INTRODUCTION

In the use of carcinostatic agents in the clinical treatment of cancer, the reports concerning acceleration or explosion of tumor growth subsequent to therapy have led to a study of the mechanisms by which these agents produce two types of effects; one inhibitory, the other stimulatory.

In 1959, Kondo and Moore[1] observed that treatment with nitrogen mustard or Actinomycin D resulted in an increase in number of metastases of mice injected with Ehrlich ascites cells intravenously. They presumed that this metastatic enhancement was mainly due to a reduction in host resistance and

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named this phenomenon as "adverse effects". Later, we[2] clarified this mechanism and found that these agents depressed antibody production to albumin in rodents and to diphtheria toxoid in man.

The causes of the adverse effects of cancer chemotherapy may be considered as follows; (1) the systemic host resistance is reduced, (2) the locally injured tissues lose some of their defences, (3) the tumor itself releases more cells into the blood stream, (4) a low concentration of drug promotes the growth of tumor cells by acting directly on the cells.

This paper is concerned with the relationship between the dosage of carcinostatic agents and tumor growth.

MATERIALS AND METHODS

Three systems were run; they are designated as animal experiment, cell culture study, and clinical study, and each will be described separately.

Animal Experiments:

Male Swiss ICR/Ha mice weighing 25–30 g and female Gifu rats weighing 90–110 g were employed. Ascites cells were obtained by harvesting after 7 days' intraperitoneal growth and washed three times with physiological saline. The viable cells were counted by staining with 0.01% Trypan Blue solution. The suspension was adjusted to contain a proposed number of cells per ml.

Schedule of Treatment; Mice were transplanted with 4.5×10^6 viable Ehrlich carcinoma cells in 0.1 ml suspension intravenously via the tail vein and treated with intraperitoneal administration of nitrogen mustard N-oxide once daily for 8 successive days starting immediately after transplantation. The survival time of mice was recorded.

Rats were transplanted with 5×10^6 viable Yoshida sarcoma cells into the right axillary space and each group of rats was treated with intraperitoneal administration of nitrogen mustard N-oxide, Mitomycin-C, or Chromomycin A_3, once daily for 7 successive days starting on the 7th day after transplantation. The animals were killed on the 21st day, the subcutaneous tumors were weighed, and the distribution and frequency of metastasis were recorded.

Tumor Cells Circulating in the Blood Stream; Rats were inoculated with 5×10^6 Yoshida sarcoma cells intravenously. Seven days later, 0.5 ml of blood was drawn from the iliac vein twice; before and after the administration of nitrogen mustard N-oxide intravenously. The living cells circulating in the blood were assayed by injecting the blood samples into both axillary spaces of new healthy recipient; the sample before injection of the drug was injected into the right and the samples after 30 min., 3 hrs., and 6 hrs. were injected into the left. Tumor "take" was observed on the 14th day after inoculation of the samples.

Microscopic examination of blood cell smears for the examination of tumor cells was run in parallel consisting of complete, rapid sedimentation of blood sample, separate centrifugation of the plasma, and the preparation of thick smears from the resulting layer of packed cells. After staining with the Giemsa solution, tumor cells were counted by comparing the number of tumor cells detected in the smears before administration of the drug with that after administration.

In Vivo and In Vitro Contact of Tumor Cells with a Drug; Rats were inoculated with 8×10^6 Yoshida sarcoma cells intraperitoneally. Seven days later, they received single intraperitoneal injection of nitrogen mustard N-oxide and were massaged gently. Three hours later, ascitic tumor cells were collected and washed with physiological saline. The counted number of unstained cells were injected into the axillary space of healthy recipients. Number of "take" was observed in the treated and control groups.

In vitro bioassay experiment was performed by contacting a small piece, adjusted in the same size, of subcutaneous Yoshida sarcoma tumor with nitrogen mustard N-oxide or Mitomycin-C. After being incubated for an hour at room temperature, the piece was transplanted into new recipient subcutaneously. Fourteen days later, the growing tumor was removed and weighed.

Cell Culture Study:

Counted number of HeLa cells were cultured with nitrogen mustard N-oxide, Mitomycin-C, or Chromomycin A_3 in 1 ml of Y.L.E. medium containing 10% calf serum. After cultivation, the cells were detached from the glass by introducing into a flask, removing the nutrient, and adding 2 ml of a 0.25% buffered solution of trypsin. Cell populations were measured by counting intact cells in a hemocytometer.

Clinical Study:

The subjects selected for study were composed of 42 patients with carcinoma of the stomach. The stomach was emptied by gastric lavage, usually the night and the next morning preceding examination. Through a Levin tube 200 ml of physiological saline in 5 divided doses were introduced into the stomach. After the patient was changed in position from supine to lateral, to face-down, gastric suction was performed before and 8 hours after the intravenous administration of nitrogen mustard N-oxide, Mitomycin-C, or Chromomycin A_3. The fluid was centrifuged immediately, and multiple smears were made of the centrifugate. May-Grünwald-Giemsa preparations were used and the number of tumor cells were classified into negative to 3 positives as graded by Papanicolaou[3].

RESULTS

Effect of Treatment with Carcinostatic Agents on the Growth of Transplanted Tumor Cells:

The survival times of mice bearing Ehrlich ascites carcinoma treated with various doses of nitrogen mustard N-oxide are shown in Fig. 1. Here, the mice given daily doses of 2.5, 0.5, and 0.25 mg per kg resulted in an early death as compared with untreated controls. With doses of 5 mg per kg, the survival times were similar to those of controls. The effects of nitrogen mustard N-oxide, Mitomycin C, and Chromomycin A_3 on the tumor growth and metastatic development of rats given inoculation of Yoshida sarcoma are shown in Table 1, in which daily treatment with 40, 4, and 0.4 μg per kg of Chromomycin A_3 that are equivalent to 1/10, 1/100, and 1/1000 LD_{50} of rat, respectively, produced much larger tumors and more frequent metastasis than those of control animals. With doses of 0.2 μg per kg corresponding to 1/2000 LD_{50} of Chromomycin A_3, the average weight of tumor and frequency of metastasis were nearly the same as those of controls. However, nitrogen mustard N-oxide produced a regression of the tumor in the doses tested.

In the treatment with Mitomycin-C, doses of 290, 29, and 2.9 μg per kg corresponding to 1/10, 1/100, and 1/1000 LD_{50}, respectively, produced a regression of the tumor, while with doses of 1.4 μg per kg corresponding to 1/5000 LD_{50}, the treatment had an adverse effect.

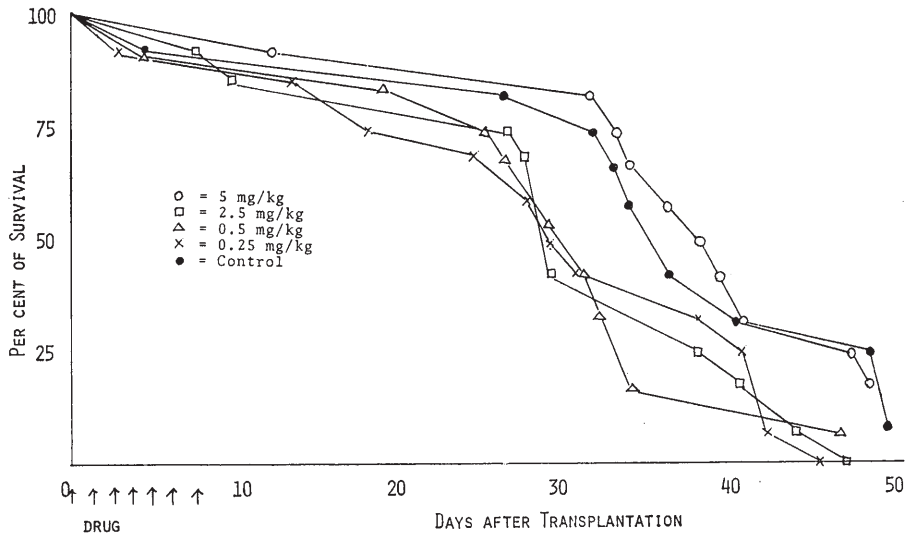

FIG. 1. Per cent survival of mice bearing Ehrlich ascites carcinoma treated with intraperitoneal injections of nitrogen mustard N-oxide and controls.

TABLE 1. Effect of Nitrogen Mustard N-oxide, Mitomycin-C, and Chromomycin A_3 on the Tumor Growth and Metastasis of Rats Given Inoculation of Yoshida Sarcoma Cells

Agent	Dose (/kg body Wt.)	No. of rats	Weight of tumor (gm)	No. of organs* with metastases	No. of organs with metastases/ No. of rats
Nitrogen mustard N-oxide	8 mg×7	12	0.30	26	2.16
	0.8 mg×7	12	0.29	33	2.75
	0.08 mg×7	11	0.63	31	2.82
Mitomycin-C	290 μg×7	11	0.00	19	1.72
	29 μg×7	16	0.68	36	2.25
	2.9 μg×7	13	1.00	28	2.15
	1.4 μg×7	13	2.12	45	3.44
Chromomycin A_3	40 μg×7	9	4.50	48	5.33
	4 μg×7	6	1.75	29	5.00
	0.4 μg×7	5	2.90	30	6.00
	0.2 μg×7	6	1.00	20	3.33
Physiological Saline		12	1.20	25	2.08

* The organs examined were local lymph glands, thoracic lymph glands, lung, heart, liver, intestine, mesentery, greater omentum, kidney, perirenals, thoracic fluid, and ascites.

TABLE 2. Bioassay of Tumor Cells in Circulating Blood Before and After Intravenous Injection of Nitrogen Mustard N-oxide

Dose (/kg body Wt.)	No. rats with tumor/No. rats injected with blood			
	Before	Interval after tumor cell inoc.		
		30 min.	3 hrs.	6 hrs.
80 mg	8/13	3/13		
	6/11		0/11	
	4/10			0/10
8 mg	6/15	3/15		
	9/27		3/27	
	1/20			4/20
0.8 mg	8/16	6/16		
	5/21		5/21	
	5/25			10/25
Physiological Saline	10/20	8/20		
	6/15		6/15	
	5/22			6/22

Assay of Tumor Cells Circulating in the Blood following Treatment of the Host with a Carcinostatic Agent:

Table 2 shows comparative tumor "take" of rats injected blood from rats that had received intravenous injection of Yoshida sarcoma cells and were treated with nitrogen mustard N-oxide. Acceleration of tumor "take" was obeserved in the rats injected blood samples from rats drawn 6 hours after administration of 8 and 0.8 mg per kg corresponding to 1/10 and 1/100 LD_{50}, respectively. With 80 mg per kg corresponding to LD_{50}, however, incidence of tumor "take" decreased. From Table 3, it is seen also that concentrations of 8 and 0.8 mg per kg produced considerable increase in number of tumor cells, while with concentrations of 80 mg per kg, the number of tumor cells decreased. The increased incidence of tumor "take" seems to be parallel to the rate of tumor cells in the blood. The treatment with low concentrations of drug may release much more numbers of cells into the blood stream.

TABLE 3. Smear-assay of Tumor Cells in Circulating Blood after Intravenous Injection of Nitrogen Mustard N-oxide

Dose (/kg body Wt.)	No. of rats	Hours after injection	%Increase	%Decrease	%Unchanged
80 mg	20	30 min.	25	50	25
	20	3 hrs.	15	60	25
	10	6 hrs.	10	60	30
8 mg	10	30 min.	30	30	40
	20	3 hrs.	45	30	25
	14	6 hrs.	50	27	23
0.8 mg	20	30 min.	45	45	10
	20	3 hrs.	45	35	20
	20	6 hrs.	40	35	25
Physiological Saline	10	30 min.	30	30	40
	10	3 hrs.	30	30	40
	10	6 hrs.	40	40	20

Assay of Tumor Cells following in vivo and in vitro Contact with a Carcinostatic Agent:

Table 4 shows the subcutaneous transplantability of the cells after intraperitoneal contact with nitrogen mustard N-oxide. A concentration of 0.8 mg per kg, tumors appeared much more than those in control animals. A concentration of 8 mg per kg, tumor appeared in only a few animals. Tumor "take" and weight of subcutaneously inoculated tumor after *in vitro* contact with nitrogen mustard N-oxide and Mitomycin-C are summarized in Table 5. A concentration of 0.5 μg per ml of nitrogen mustard N-oxide or Mitomycin-

TABLE 4. Subcutaneous Transplantability of Tumor Cells after Intraperitoneal Contact with Nitrogen Mustard N-oxide

Dose (/kg body Wt.)	Cell number transplanted*	
	10^3	10^4
8 mg	1/10	3/10
0.8 mg	9/15	13/16
Control	6/18	13/21

* No. rats with tumor/no. rats injected with tumor cells.

TABLE 5. Tumor "Take" and Weight of Subcutaneously Inoculated Yoshida Sarcoma after *In Vitro* Contact with Nitrogen Mustard N-oxide and Mitomycin-C

Agent	Dese (/ml)	No. "Take"	Mean tumor Wt. (g)
Nitrogen mustard N-oxide	50 μg	1/10	0.1 (0, 0, 0, 0, 0, 0.5, 0, 0, 0, 0)
	5 μg	6/10	0.9 (0.7, 0, 2, 2.5, 2.5, 1.5, 0.5, 0, 0, 0)
	0.5 μg	8/10	1.7 (1.0, 1.5, 0, 3.5, 3.5, 0, 3.5, 0.5, 2.5, 1)
	0	9/10	1.0 (1, 1.5, 1.5, 2, 1, 0, 0.5, 1, 1, 1)
Mitomycin-C	50 μg	3/10	0.2 (0, 0, 0.5, 0, 0, 0.7, 0.5, 0, 0, 0)
	5 μg	7/10	0.8 (1, 1.5, 1, 0, 1, 0, 0, 1.5, 1, 1.7)
	0.5 μg	9/10	2.0 (1, 2, 2, 0, 2.5, 2, 1.7, 2.5, 3, 3.5)
	0	9/10	1.0 (1, 1.5, 1.5, 2, 1, 0, 0.5, 1, 1, 1)

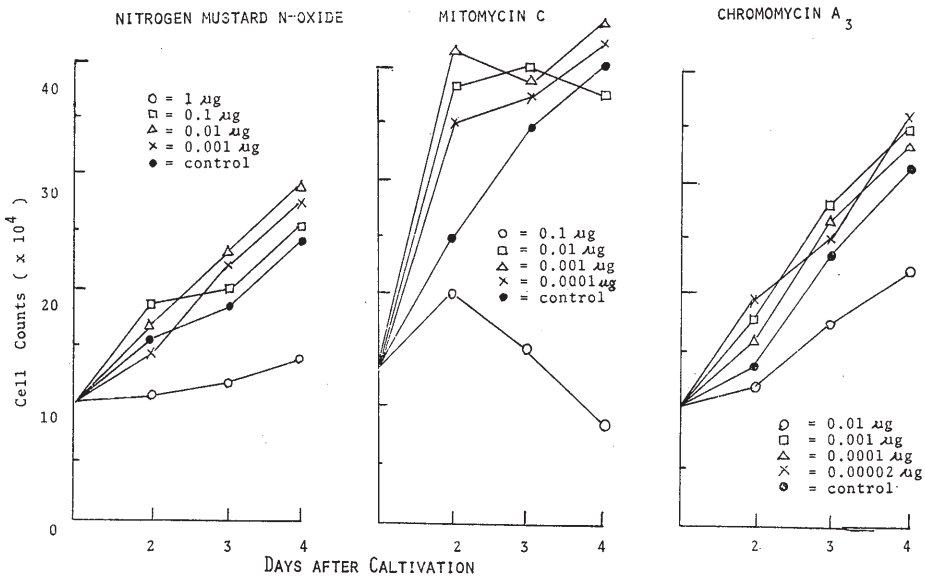

FIG. 2. Effect of carcinostatic agents on the growth of HeLa cells.

C, tumor attained a much greater weight than those in the control animals.

Effect of Carcinosiatic Agents on the Growth of Cultured Cells:

Fig. 2 shows the effect of each concentration of drug on the growth curve of HeLa cells. It is clear that high concentrations of these drugs inhibited the cell growth, while low concentrations showed much larger number of cells than those of untreated controls. A concentration of 0.001 μg per ml of

TABLE 6. Comparison of Washes of Stomach Cavity Before and after Injection of Carcinostatic Agents

Agent	Case No.	Extent of tumor cells*	
		Before	After
Nitrogen mustard N-oxide	1	+	++
	2	−	−
	3	+	++
	4	−	−
	5	−	+
	6	−	−
	7	−	+
	8	+	++
	9	−	++
	10	+	+++
	11	+	+
	12	+	+
	13	+	+
	14	+	++
	15	+	++
	16	+	++
	17	−	+
	18	++	−
	19	+	++
	20	+	++
Mitomycin-C	1	+	++
	2	+	++
	3	−	−
	4	±	±
	5	−	−
	6	++	+++
	7	++	+++
	8	+	−
	9	+	++
	10	−	−
	11	±	±
	12	+	++
	13	+	++
	14	−	+
Chromomycin A_3	1	+	+
	2	−	−
	3	±	+
	4	+	−
	5	−	+
	6	−	−
	7	+	++
	8	++	+++

* Graded by Papanicolaou's criteria.

Mitomycin-C showed prominent evidence of this phenomenon.

Cancer Cells Released into the Stomach Cavity of Patients with Carcinoma of the Stomach after Intravenous Injection of Carcinostatic Agents:

Washings obtained from patients with carcinoma of the stomach before and after injection of nitrogen mustard N-oxide, Mitomycin-C, and Chromomycin A_3 are compared in Table 6. The results obtained in this study indicate that cancer cells released into the stomach cavity were increased by the administration of these drugs. Of the 20 patients, 13 had much larger number of cells after a single injection of nitrogen mustard N-oxide in a dose of 50 mg. Four patients with negative tumor cells turned positive after the injection. In the treatment with Mitomycin-C in a dose of 2 mg, 8 of 14 patients had increased number of cells after the injection. One negative patient turned positive. In the treatment with Chromomycin A_3 in a dose of 0.5 mg, 4 of 8 patients had increased tumor cells after the injection. As contrast, negative to negative or positive to negative tumor cells after treatment was observed in a few instances.

DISCUSSION

Foregoing data[1)2)4)] have demonstrated that tumor growth and metastases are at one time stimulated and at another time inhibited by the same drug depending on difference in time and duration of administration. Kondo and Moore[1)] have shown that the maximum number of metastases occurred in mice given two injections of nitrogen mustard and Actinomycin D but with larger doses showed a true chemotherapeutic effect. Later, Kondo and Ichihashi[2)] showed that an increase of metastasis formation was observed when the host was treated with the agent before inoculation of tumor cells or treatment was started at a later stage of tumor development.

The present study demonstrates the presence of both growth-stimulating and growth-inhibiting actions of the same drug depending only on a difference in dosage. Nitrogen mustard N-oxide, Mitomycin-C, and Chromomycin A_3 tested in this study were found to stimulate the growth of tumor cells and cultured cells both *in vivo* and *in vitro* in low concentrations. Abd El-Ghaffar[5)] reported that triethylene-thiophosphoramide and 5-fluorouracil in relatively small doses were found to stimulate the growth of sarcoma 180 both *in vitro* and *in vivo*. Kayama *et al.*[6)] also observed increased number of Ehrlich ascites carcinoma cells when the host was treated with low concentrations of Mitomycin-C.

In our previous report[2)], it has been demonstrated that acceleration of tumor growth was due to decrease of host resistance. Larger doses must cause much depressed host resistance and increased tumor growth and metastasis.

The present study shows that smaller doses stimulated tumor growth as well, probably indicating a different mode of action. The mode of the stimulating action is not clear though it is evident that it is a direct action on the cells since it was demonstrated *in vitro.*

On the cause of metastasis formation, two reasons may be considered; one is much larger number of cells released into the blood stream following direct action of carcinostatic agents as indicated in this study, the other is increase in their capacities of adhesiveness to the blood vessels following the damage of the vessels by the administration of the agents as indicated by Wood[7].

We find here one of the dilemmas of present day cancer chemotherapy. Large doses must, of course, be necessary to produce the maximum effect, but they depress, in varying degrees, host resistance and this may sometimes impair their effectiveness. Small doses have also a stimulating action on neoplastic growth. In the clinical study, the dosage used clinically produced much more cells into the stomach cavity. It suggests the danger that carcinstatic agents may sometimes stimulate neoplastic growth and should always be taken into consideration in the treatment of cancer patients.

REFERENCES

1) Kondo, T. and Moore, G. E., The limitations and adverse effects of cancer chemotherapy, *Surgical Forum*, **9**, 607, 1959.
2) Kondo, T. and Ichihashi, H., Induction of Metastases by treatment with carcinostatic agents. II. Depression of host resistance and antibody production, *GANN*, **55**, 403, 1964.
3) Papanicolaou, G. N. and Cooper, W. A., The cytology of the gastric fluid in the diagnosis of carcinoma of the stomach, *J. Nat. Cancer Inst.*, **7**, 357, 1947.
4) Kondo, T. and Moore, G. E., Production of metastases by treatment with carcinostatic agents. I. Effects of carcinostatic agents on the host, *Cancer Res.*, **21**, 1396, 1961.
5) Abd El-Ghaffar, Y., The growth-stimulating effect of TSPA and 5-FU on the cells of sarcoma 180, *Cancer*, **16**, 8, 1963.
6) Kayama, T., Shiomi, F., and Onoda, H., Studies on an oncostatic agent "K.C.G." (V), *Proc. Japan. Cancer Assoc.*, 102, 1964.
7) Wood, S., Jr., Holyoke, E. D., and Yardley, J. H., Mechanisms of metastasis production by blood-borne cancer cells. In Proceedings of the Fourth Canadian Cancer Research Conference, New York, N. Y. Academic Press Inc., pp. 167, 1961.