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Screening Test for the Prevention of Metastases produced by Ehrlich Carcinoma Cells[1]

Yoshinari Hasegawa, Tsutomu Irikura[2a] and Den-ichi Mizuno[2b]

Kyorin Chemical Laboratory[2a] and Faculty of Pharmaceutical Sciences, University of Tokyo[2b]

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A screening of some substances for antimetastasis effect was carried out. The test system has previously been reported. The LP-12 line of Ehrlich ascites carcinoma cells were inoculated into the tail vein of ddY mice to give pulmonary tumor foci reproducively.

Drugs to be tested were injected simultaneously with the tumor inoculation. Among some known antitumor drugs, bleomycin and cyclophosphamide have shown excellent effects. Non-ionic surface active agent, Triton X-100 also has been shown to give a slight activity.

Introduction

In the cancer chemotherapy, intravenous transplantation of tumor cells into experimental animals has been used in several laboratories[3-5] as a model system for tumor metastasis. The results, however, are not consistent with each other for survival days of the host animal when Ehrlich ascites carcinoma cells were employed. A previous report[6] showed the establishment of LP-12 subline from Ehrlich ascites carcinoma cells for a tool of the screening of antimetastatic activity. The mice when inoculated intravenously with 6×10^6 cells of this established line died reproducibly in less than 20 days due to the incidence of lung tumor. Therefore, it can be used routinely for the screening of drugs for antimetastasis, since the results are highly reproducible and easily obtainable by observing the survival time of mice within a short period. The present report summarizes the results of some screening data.

Experimental

The animals used throughout this experiment were ddY male mice, weighing 20—23 g. They were fed with semi synthetic diet CE-2 from CLEA Japan Inc., Tokyo and given water *ad libitum*. The mice were supplied from an animal farm in Shizuoka prefecture. Ehrlich ascites carcinoma cells were used as the LP-12 cell line which have been maintained by serial intraperitoneal transplantation into ddY male mice.[6] Seven days old tumor cells were employed as a material for the experiment. The solution of 6×10^6 tumor cells in 0.2 ml of $NaHCO_3$-free Tyrode was transplanted intravenously into the tail vein of mouse. The drugs to be tested were given in a saline solution of 0.25 ml intraperitoneally or intravenously. Six mice in a group are usually employed for each assay. The survival of mice were observed for 30 days. Effectiveness of drugs against the metastasis was judged from the survival time and the pathological observation.

1) Presented at Proceedings of the 27th and 28th General Meeting of the Japanese Cancer Association, Tokyo, October 1968 and Kanazawa, October 1969.
2) Location: *a*) *Ukima, Kita-ku, Tokyo*; *b*) *Hongo, Tokyo*.
3) Y. Hayashi, Y. Shirasu and F. Fukuoka, *Gann*, **51**, 335 (1960).
4) S. Wood, Jr., E.D. Holyoke and J.H. Yardley, *Canad. Cancer Conf.*, **4**, 167 (1961).
5) D. Agostino and E.E. Clifton, *J. Surg. Res.*, **2**, 343 (1962).
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Results

Table I summarizes the screening data of antimetastatic effect of some well known antitumor drugs so far tested. The drugs were given simultaneously at the time of the tumor cell inoculation, followed by daily administration of the drug for 7 days. When these drugs were administered at the onset 90 minutes prior to or 24 hours after the tumor inoculation, all the drugs tested showed essentially the same results as in the case of simultaneous administration. Bleomycin among these test compounds shows a marked activity. Phospholipase A

Table I. Screening Results of Some Antitumor Drugs for Antimetastasis Effect

Drug[a]	Dose (mg/kg)	Route	S/T[b]	Survival day mean ± S.C. (T/C)[c]	Percent (T/C)[c]
Bleomycin	7×7	*i.p.*	3/6	24.0±6.4/11.3±2.4	212
Mitomycin C	2×7	*i.p.*	0/6	14.5±2.6/10.0±2.4	145
6-Mercaptopurine	40×7	*i.p.*	0/6	14.0±2.0/10.0±2.4	140
5-Fluorouracil	30×7	*i.p.*	0/6	10.7±3.6/11.2±3.8	96
5-Fluorouracil	4×7	*i.p.*	0/6	9.2±1.9/11.3±2.4	81
Nitromin	4×7	*i.p.*	0/6	10.8±1.1/11.0±2.7	98
Cyclophosphamide	100×7	*i.p.*	0/6	11.2±3.2/11.2±3.8	100
Cyclophosphamide	15×7	*i.p.*	0/6	14.2±2.7/11.3±2.4	125
4,6-DNQO	40×7	*i.p.*	0/6	13.3±0.7/10.0±2.4	133
Hydrocortisone	4×7	*i.p.*	0/6	11.7±1.8/11.0±2.7	106

Each mouse was inoculated intravenously with 6×10^6 cells of LP-12 line of Ehrlich carcinoma. The drug was injected simultaneously at the time of cell inoculation, followed by daily dosage for 7 days.

a) nitromin: 2,2′-dichloro-N-methyldiethylamine hydrochloride; cyclophosphamide: 1-bis(2-chloroethyl) amino-1-oxo-2-aza-5-oxaphosphoridin; 4,6-DNQO: 4,6-dinitroquinoline-1-oxide

b) S/T: survivours for over 30 days per the total numbers treated

c) T/C: Ratio of the treated to the control Survivor for over 30 days was calculated as days survival.

Essentially the same results were obtained, when these drugs were given at the onset 90 minutes prior to or 24 hours after the tumor transplantation followed by the daily dosage for 7 days.

Table II. Screening Results of Some Antitumor Drugs for Antimetastasis Effect

Drug[a]	Dose (mg/kg)	Route	S/T	Survival day mean ± S.C. (T/C)	Percent (T/C)
Cyclophosphamide	120	*i.p.*	2/6	22.1±10.1/11.8±1.8	175
Bleomycin	50	*i.p.*	1/6	14.0± 7.0/ 9.1±1.6	154
Myelobromol	1000	*i.p.*	1/6	14.5± 7.0/10.7±3.1	136
5-Fluorouracil	30	*i.p.*	1/6	15.2± 4.7/11.2±3.8	136
Methotrexate	20	*i.p.*	0/6	14.1± 5.4/11.2±3.8	126
6-Mercaptopurine	40	*i.p.*	0/6	10.1± 1.7/ 9.1±1.6	111
Vinblastin	2.5	*i.p.*	0/6	10.2± 1.8/10.1±1.0	101
Mitomycin C	2	*i.p.*	0/6	9.7± 0.7/10.1±1.0	96
Nitromin	4	*i.p.*	0/6	9.5± 0.9/10.1±1.0	94
Hydrocortisone	4	*i.p.*	0/6	9.2± 1.0/10.1±1.0	91
4,6-DNQO	40	*i.p.*	0/6	8.8± 1.2/10.1±1.0	87
Degranol	10	*i.p.*	0/6	8.6± 1.3/10.1±1.0	85
Cyclophosphamide	120	*i.v.*	5/6	28.7± 3.3/11.8±1.8	240
Bleomycin	50	*i.v.*	1/6	21.8± 6.8/11.3±2.4	193
Mitomycin C	2	*i.v.*	1/6	16.2± 6.6/10.7±3.1	151
Vinbrastin	2.5	*i.v.*	0/6	11.8± 2.1/10.7±3.1	110
Degranol	10	*i.v.*	0/6	11.2± 1.9/10.7±3.1	105
5-Fluorouracil	30	*i.v.*	0/6	9.2± 1.9/11.3±2.4	81

Experimental conditions are the same as in Table I, eaxcept that the drug was given once simultaneously with the tumor inoculation.

a) myelobromol: 1,6-dibromo-1,6-dideoxy-D-mannitol; methotrexate:N-[*p*-{[(2-amino-4-hydroxy-6-pteridinyl) methyl]methylamino}benzoyl]glutamic acid; degranol: 1,6-bis(2-chloroethylamino)-1,6-dideoxy-D-mannitol dihydrochloride

(8 μg/kg, ×7) from snake venom was also tested by the same method, and lipopolysaccharide (4 mg/kg, ×3) of *Escherichia coli* was examined with intracutaneous injection according to the method of Mizuno, *et al.*[7] However, the results have shown the data, 106% and 110%, respectively. It means they have no effect.

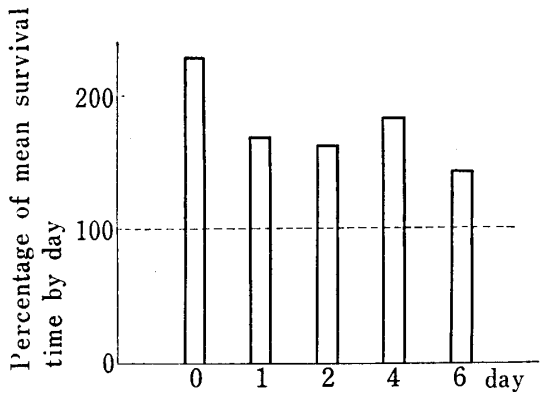

Fig. 1. Effect of Single Intraperitoneal Injection of Cyclophosphamide after the Intravenous Inoculation of Ehrlich Carcinoma Cells

Each mouse was inoculated intravenously 6×10^6 tumor cells. Drugs was given in a dose of 100 mg/kg once at 0, 1, 2, 4 and 6 days after the tumor inoculation and each group (6 in one group) was observed for 30 days. Percentage of mean survival time by day is calculated as indicated in Table I. Dotted horizontal line at 100% shows the control value.

We attempted to give a single injection of drugs to the animals intraperitoneally or intravenously to minimize the side effect of the drugs. The drugs were given at the simultaneous time of the tumor inoculation. Table II shows the results. The single-dose administration of each drug appeared to be more or less effective against tumor metastasis. Mitomycin C, cyclophosphamide and 5-fluorouracil showed better effect than in the case of 7 consecutive injections of Table I. Especially, cyclophosphamide gives the most excellent effect compared with other compounds so far tested. However, tumor foci in the lung tissues were observed even though the mice survived for over 30 days.

Fig. 1 shows the results of the activity of cyclophosphamide when the drug was administered at different times. The most effective time of the injection was at the simultaneous time of the tumor inoculation.

Many other compounds were tested for the prevention of metastases with the same test system above mentioned. Most compounds tested were injected intravenously (Table III).

Table II. Screening Results of Some Compounds for Antimetastasis Effect

Compound[a]	Dose (mg/kg)	Route	S/T	Survival day means S.C. (T/C)	Percent (T/C)
Heparin	40	*i.v.*	0/6	11.2±0.9/ 9.5±1.3	118
Dextran	500	*i.v.*	0/6	15.5±2.4/11.8±1.8	131
Benzalkonium chloride	10	*i.v.*	0/6	11.8±2.6/ 9.5±1.3	124
Sodium lauryl sulfate	10	*i.v.*	0/6	10.5±2.8/ 9.5±1.3	111
Triton X-100	10	*i.v.*	1/6	19.3±6.6/10.7±3.1	180
Triton WR-1339	10	*i.v.*	0/6	12.8±1.9/11.6±1.7	110
Tween 80	10	*i.v.*	0/6	11.0±4.0/11.0±3.5	100

Experimental conditions are the same as in Table II.
a) dextran: average molecular weight, 54000; triton X-100: polyoxyethylene-iso-octylphenol ether; Triton WR-1339: polyoxyethylene octylphenol ether; Tween 80: polyoxyethylene sorbitan monooleate

Among them some surfactants were tested in an attempt to inhibit the attachment of the tumor cells to the vena in the lung. Triton X-100 was slightly effective, but other non-ionic surfactants, polyoxyethylene- or polyoxyethylene-sorbitan-derivatives were not effective on our unpublished data. When the cell suspension in $NaHCO_3$-free Tyrode was incubated with 0.1% of each detergents at room temperature for 20 minutes, the viability of tumor cells was tested by growth of the cells transplanted in the peritoneal cavity of normal mice after the treatment. Other detergents exert no effect on the viability.

As shown in Table IV, tumor cells are considered to die irreversibl after the treatment with 0.1% solution of Triton X-100, since the permeability of the cells increased greatly as

7) D. Mizuno, O. Yoshioka, M. Akamatsu and T. Kataoka, *Cancer Res.*, **28**, 1513 (1968).

Table IV. The Viability as Estimated by PSB Uptake of Tumor Cells after the Treatment with Triton X-100

Concentration of Triton X-100 (%)	Viability of tumor cells[a)]	PSB uptake[b)] (OD at 630 mμ)
0	yes	0.07
0.01	yes	0.07
0.1	no	0.27

LP-12 line of Ehrlich carcinoma(10^6 cells) were stirred mildly in $NaHCO_3$-free Tyrode solution of Triton X-100 at room temperature (22°) for 20 minutes.
a) Judged from transplantability into the peritoneal cavity of mice after the treatment.
b) According to the technic described by Nishino, *et al.*[8] PSB; Pontamine sky blue 6B or Brilliant blue 6B

revealed by the uptake of pontamine sky blue 6B according to the technic described principally by Nishino, *et al.*[8] Thus it can be considered that a marked alteration in cell permeability is brought about by the presence of Triton X-100 in suspensions of Ehrlich cells. Since Triton X-100 is not so toxic, this suggests that the action will throw light on the approach to the chemotherapy for tumor-metastasis. However, the synergistic effect of Triton X-100 in combination with other antitumor agents were not increased in our test system.

Discussion

As shown in Table I, the present results can be evaluated as a model system for the screening of substances with antimetastatic activity. Antitumor agents known as ineffective against metastasis have shown negative results, whereas cyclophosphamide which is known as effective against metastasis has more or less positive results.

In Table I some new findings have been indicated. Among them the result of bleomycin is to be noted. According to Umezawa[9] accumulation of bleomycin to lung tissue of mice is to be marked and our results also have coincided with theirs. However, it remains obscure as to whether it is really due to the preferential accumulation of bleomycin in tumor foci of the lung.

Single-dose injection of cyclophosphamide showed an excellent effect to prevent the metastases (Table II and Fig. 1). In other experimental tumor systems by Karrer, *et al.*,[10] continuous long-term cyclophosphamide-treatment was proved to be ineffective due to the eventual development of lethal toxicity. A single-dose administration of the drug appears to be equally effective against pulmonary matastases by their test system. Mitomycin C was ineffective although the drug shows a remarkable effect against ascites form of the Ehrlich tumor. It must be considered that the drugs have preferential specificity to the type of tumors and to the tissue of hosts.

Wood, *et al.*[4] published an extensive review concerning mechanism of metastasis from blood-borne tumor cells. In their reports, heparin, dicumarol and plasmin for anticoagulants have been described to be effective in preventing metastasis. Heparin effected a mitotic inhibition on the Ehrlich ascites carcinoma *in vivo*,[11] and was far more effective than dextran for the prevention of a coagulation thrombus.[12] The action of low molecular weight-dextran

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9) H. Umezawa, The 6th Congress of Japan Society for Cancer Chemotherapy, Tokyo, October 1968.
10) K. Karrer, S.R. Humphreys and A. Goldin, *Int. J. Cancer*, **2**, 213 (1967); K. Karrer and S.R. Humphreys, *Cancer Chemother. Rep.*, **51**, 439 (1967).
11) S.M. Lippman, *Cancer Res.*, **17**, 11 (1957).
12) V. Gurewich and D.P. Thomas, *J. Lab. Clin. Med.*, **66**, 602 (1965).

on blood coagulation and adhesiveness to cells is similar to that of heparin. Some workers[13] have observed a decreased incidence of lung metastases following dextran administration, whereas Wood, *et al.*[14] failed to prevent the pulmonary metastases by dextran. On the other hand, the administration of dextran resulted in an progressive increase of hepatic metastases in rabbit which were received intraperitoneal inoculation of V2 carcinoma cells.[15] As far as our results are concerned, heparin and dextran are completely ineffective (Table III). These contradicts will require further experimental evaluation prior to clinical trials of these compounds to patients.

It has been reported that permeability of the membrane of Ehrlich tumor cells increases considerably when a surface active agent, Tween 60 or Tween 80, is added to the incubation medium.[16,17] According to Palmer, *et al.*[18] nigrosin-uptake is proved to be the most satisfactory indicator for the activity of surfactant on tumor cells. They observed morphological changes in the Ehrlich ascites cells treated with surfactants (anionic, cationic or non-ionic detergent). Holmberg[19] summarized the characteristic of different stages of cell-injury leading to death. They also confirmed that undamaged live cells are impermeable to lissamine green.

Triton X-100 is a typical lysosomal labilizer. A combined therapy with lysosomal labilizers (vitamin A, testosterone or plasmin) and antitumor agents (cyclophosphamide or mitomycin C) to accelerate the cytotoxic effect have been performed by some workers.[20] However, a synergistic effect of antimetastasis was not obtained in our experiment when the combination of Triton X-100 and some antitumor drugs were employed. This contradict remains to be ascertained.

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15) B. Fisher and E.R. Fisher, *Cancer Res.*, **28**, 1586 (1968).
16) E.R.M. Kay, *Cancer Res.*, **25**, 764 (1965).
17) A.G. Malenkov, S.A. Bogatyreva, V.P. Bozhkova, E.A. Modjanova and J.M. Vasiliev, *Exptl. Cell Res.*, **48**, 307 (1967).
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