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STUDY ON THE SENSITIVITY TEST OF CARCINOSTATIC AGENTS BY ACID PHOSPHATASE ACTIVITY

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ABSTRACT

A new method of tumor sensitivity test by measurement of the activity of acid phosphatase was presented.

Treatment of experimental tumor cells with mitomycin C, chromomycin A_3 and 5-fluorouracil showed that deviation index of the specific activity ran well parallel with viability of the cells *in vitro*. The deviation index of tumor cells was also in agreement with survival times of tumor bearing mice treated with these drugs. Side effects appeared in animals having high values of deviation indices of liver cells. The effectiveness of the drugs was expressed by the values obtained from tumor cells minus those obtained from liver cells. In case of cyclophosphamide, however, it was difficult to judge by this method.

In the therapy with mitomycin C, the response was well correlated with the deviation index of tumor cells and the side effect was also correlated with the deviation index of liver cells.

This method could predict the effectiveness of carcinostatic agent especially short acting cytocidal drugs.

INTRODUCTION

Up to the present, many carcinostatic agents have been used in cancer chemotherapy, but their effects are not enough for patients with malignancy. The reason why no satisfactory results are obtained in cancer chemotherapy may be that effective drugs are also toxic to the host and the drugs have been used at random. For this reason, the selection of a proper agent, which is most effective to tumor cells as well as ineffective to normal host cells, is quite important in the treatment of patients with neoplasm. Various methods for selecting the drugs, *i.e.* cylinder plate method[1], cell agar plate (CAP) method[2] or agar plate diffusion technique[3)~7)], tissue culture method[8)~11)], succinic dehydrogenase inhibition (SDI) test[12] and so on[13)~16)] have been reported, but no perfect method for selection has yet been established. For the purpose of finding a new method of screening of the drugs in cancer chemotherapy,

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the present study was designed on the fact that lysosomal enzymes play an important role in the degenerative processes of cells[17)~23)]. Of the lysosomal enzymes, the activity of acid phosphatase was measured on both transplanted tumor cells and patients with cancer.

MATERIALS AND METHODS

A) Animals

a) Mice; ICR Swiss male, dd male, C 57 BL male, CBA male and C 3 H/He female mice were delivered from an inbred colony of the laboratory animal center, Nagoya University School of Medicine, maintained by strict brother and sister mating for years. The weights of mice used ranged from 20 to 24 g.

b) Rats; M. P. male rats having marker gene "ccaaBBhh" and weighing about 100 g were obtained from the Moriyama Psychiatory Hospital*.

B) Tumors

The following tumors were used: A hyperdiploid Ehrlich ascites tumor maintained in ICR Swiss mice by successive intraperitoneal implantation of 5×10^6 cells every 6 days. Yoshida sarcoma (nitrogen mustard N-oxide sensitive strain) obtained from Aichi Cancer Center in Nagoya, AH-130 and Friend virus from National Cancer Center in Tokyo, and FM 3 A tumor from Mie University in Tsu.

C) Carcinostatic agents

The agents used were all commercial products.

a) Antibiotics: mitomycin C (MMC) purchased from Kyowa Fermentation Industry, Ltd., Tokyo; chromomycin A_3 (CMA) from Takeda Chemical Industry, Ltd., Osaka.

b) Alkylating agents: cyclophosphamide (CPA) purchased from Shionogi Pharmaceutical Company, Ltd., Osaka; nitrogen mustard N-oxide (NMO) from Yoshitomi Pharmaceutical Company, Ltd., Osaka.

c) Antipyrimidine: 5-fluorouracil (5 FU) purchased from Kyowa Fermentation Industry, Ltd., Osaka.

d) Antimetabolite: methotrexate (MTX) purchased from Takeda Chemical Industry, Ltd., Osaka.

e) Plant extract: vincristin purchased from Shionogi Pharmaceutical Company, Ltd., Osaka.

D) Cell suspensions

a) Tumor cell suspension: Ascites tumor cells obtained from the intra-

* Adress; 6, Kitayama, Moriyama, Nagoya, JAPAN.

peritoneal cavity were diluted to contain approximately 2×10^7 cells per ml by adding 1/15 M phosphate buffered saline pH 7.2 (PBS). Solid tumor was cut into small pieces with sharp scissiors, minced with tissue crusher[12], suspended in sterile PBS and filtered through four layers of gauze. The cell suspension thus obtained was diluted to contain approximately 2×10^7 cells per ml of PBS.

b) Liver cell suspension: The cell suspension of liver tissue was prepared by the same procedure as described above.

E) Chemicals

Disodium p-nitrophenyl phosphate purchased from Katayama Chemistry Co. Ltd., Nagoya. Buffer consisted of 0.075 M sodium succinate and 0.025 M succinic acid and was adjusted to pH 5.0 with pH meter.

F) Assay of enzymatic activity

To the cell suspension divided into several tubes of 0.2 ml each was added 0.2 ml of the proposed concentration of carcinostatic agents in PBS and 1.6 ml of PBS. The mixture was incubated for 3 hours at 37°C in an incubator and was shaken several times. After centrifugation, the supernatant was discarded and the sediment was prepared in a cold water bath for determination of acid phosphatase activity. A wide variety of assay procedures have been used for acid phosphatase[24)-27)]. The method of Lowry *et al.*[24)], in which the hydrolysis of p-nitrophenyl phosphate was followed by spectrophotometric determination of the free p-nitrophenol, was popular and convenient.

In this experiment, Lowry's method was slightly modified as follows for simplicity in laboratory conditions. The enzyme activity was measured at 37°C with a final volume of 2 ml of bufferrd cell suspension at pH 5.0 containing 16 mM disodium p-nitrophenyl phosphate. Fifteen minutes later, the reaction was stopped by adding 0.5 ml of 30 percent trichloroacetic acid solution. The contents were then centrifuged at 3,000 rpm for 5 min. (or 10 min. more when necessary) and 1.6 ml of the supernatant was gently poured into 2.5 ml of 1.5 N NaOH. The released p-nitrophenol was then measured by the Hitachi Perkin-Elmer UV-VIS spectrophotometer at 410 mμ. The precipitated protein was determined by the method of Lowry *et al.*[28)].

G) Evaluation of in vitro experiments

a) Deviation index (D.I.) as a parameter of the effect of the agents was calculated by the following formula;

$$\text{D.I.} = \frac{P/Q}{P'/Q'} \times 100 - 100,$$

where P: p-nitrophenol released by acid phosphatase in mM
Q: quantity of protein in mg
these values obtained from tumor cell suspension with agents
P', Q': value obtained from tumor cell suspension alone

b) The cells were incubated with the agents for 3 hours and the number of trypan blue unstained cells was counted with a hemocytometer. The percentage effect was calculated by the following formula[29];

$$\% \text{ effect} = 100\left(1 - \frac{A}{C}\right),$$

where A: number of unstained cells in tumor cell suspension with agent
C: number of unstained cells in tumor cell suspension alone

H) In vivo experiments

a) Ascites form of the tumor: The experimental animals were inoculated intraperitoneally with 5×10^6 viable ascitic cells; Ehrlich, FM 3 A, and Friend virus induced tumor were inoculated into mice and AH-130 and Yoshida sarcoma into rats. They were divided into several groups of 10 animals each. Twenty-four hours later, they were injected daily with 0.04 μg/g of body weight of MMC, 2 μg/g of CPA, 0.01 μg/g of CMA, 5 μg/g of 5 FU, 0.1 μg/g of MTX and 0.02 μg/g of vincristin dissolved in 0.2 ml of PBS, for 7 successive days. One group of animals surved as untreated control. Survival times of the animals were observed and statistical significance was calculated by the t-test.

b) Solid form of the tumor: Fifty animals were inoculated subcutaneously with 1×10^6 tumor cells. One week later, the animals with solid tumors of sizes about 7–9 mm in diameter were divided into five groups of 10 animals each and were treated intraperitoneally with 0.4 μg/g of MMC, 20 μg/g of CPA, 0.1 μg/g of CMA and 50 μg/g of 5 FU in 0.2 ml of PBS and 0.2 ml of PBS as control, for 5 days. The size of the tumor was measured at least along two directions by a caliper.

c) Toxicity of the agents on the host: ICR Swiss, dd, C 57 BL, and CBA strains of mice were injected intraperitoneally in a dose of 1 μg/g of MMC, 50 μg/g of CPA, 0.25 μg/g of CMA and 125 μg/g of 5 FU in 0.2 ml of PBS every day, until the animals died. The toxicity of the agents on the host was recorded by the survival time of the mice.

d) Observation of side effects of MMC: Four strains of mice were injected intravenously with a dose of 5 μg/g twice, 4 days apart. They were checked survival times, for leukocyte counts and albuminuria (using Uristix from Ono Drug Company in Osaka) every other day.

I) Clinical responses

The deviation index of biopsied tumor from inoperable patients with malignant disease were measured. These patients were treated with appropriate drugs and their clinical responses were compared to the deviation indices. Twenty-five patients with carcinoma of the stomach were treated with MMC during curative radical surgery or later. Side effects including leukocyte

count, s-GOT and s-GPT, were compared with the deviation indices of the liver biopsied at surgery. Furthermore, nineteen patients with carcinoma of the stomach treated by curative radical surgery plus MMC chemotherapy were followed up. The relationship between (T) − (L) values and survival times was observed.

RESULTS

A) Preliminary experiments

a) Effect of pH on activity of acid phosphatase: Between pH 4.0 and 6.0 succinate buffer was examined for the assay of acid phosphatase. As shown in Fig. 1, the optimal pH for Ehrlich ascites tumor cells was between 4.8 and 5.4, and in the succeeding experiments, pH 5.0 succinate buffer was utilized.

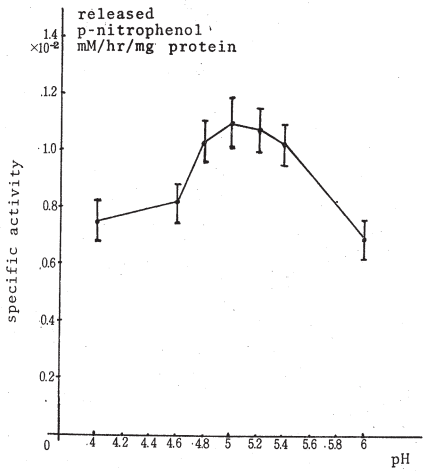


FIG. 1. Effect of pH on the specific activity of acid phosphatase.

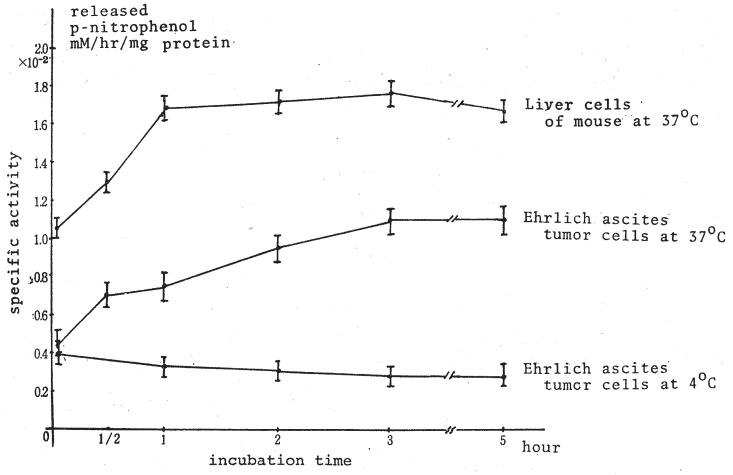


FIG. 2. Effect of incubation time on the specific activity of acid phosphatase.

b) Effect of incubation time on activity of acid phosphatase: The curve in Fig. 2 shows the relationship of specific activity of acid phosphatase of Ehrlich ascites tumor and incubation time. The activity of enzyme of the tumor cells increased with the time of incubation at 37°C and became stable 3

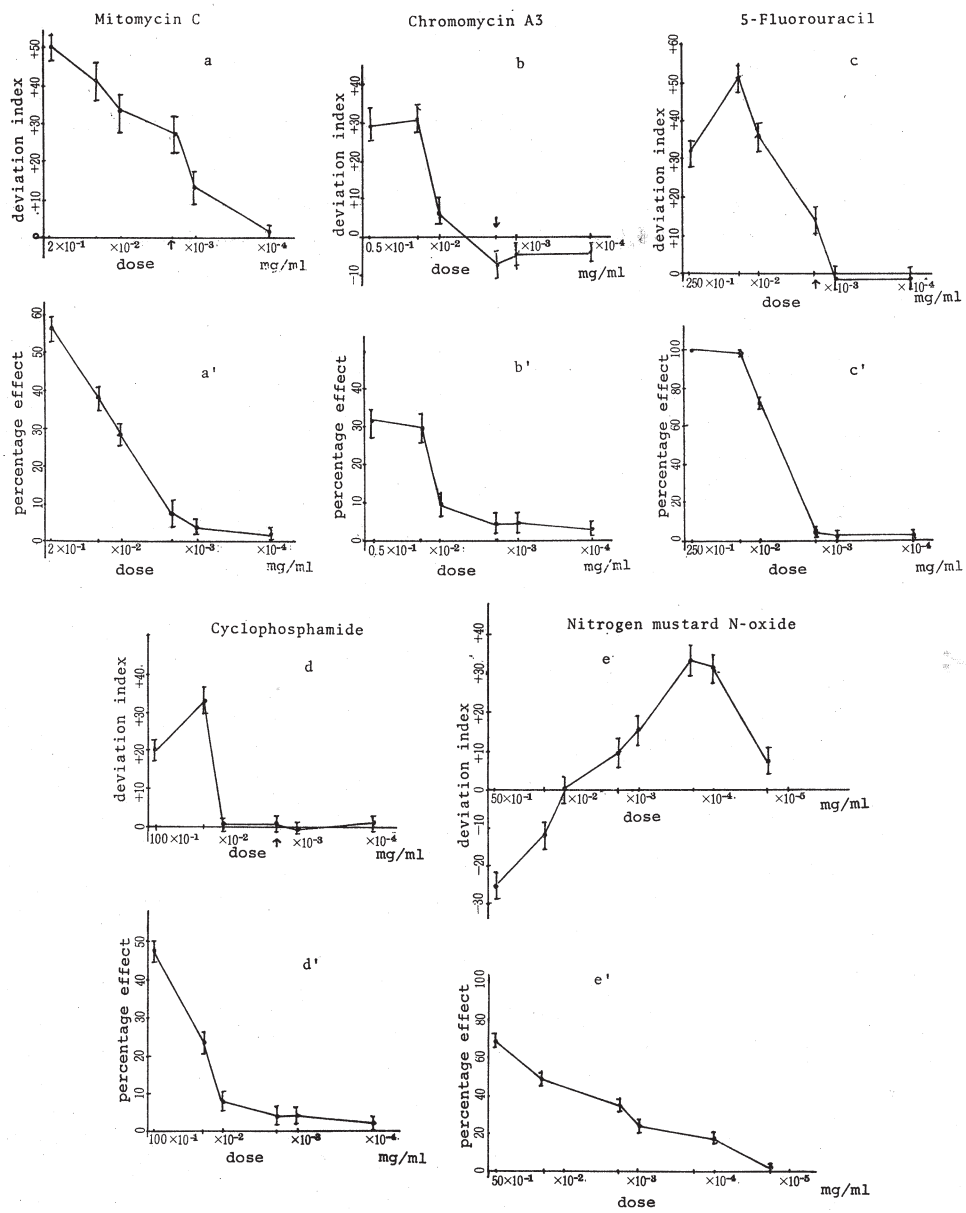
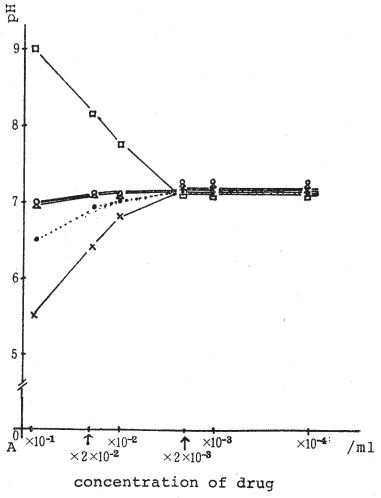


FIG. 3. Deviation index curve and percentage effect curve of Ehrlich ascites tumor cells.

hours later, whereas, it remained constant during incubation at 4°C. Activity of the enzyme of liver cells showed also the same curve as that of Ehrlich tumor cells.

c) Factors affecting the deviation index of Ehrlich ascites tumor cells treated with various carcinostatic agents: Figs. 3 a–e show the relationship between deviation index of the tumor and concentration of carcinostatic agents. Arithmatic scale of deviation index was used on the ordinate and logarithmic scale of doses of the agents per one ml on the abscissa. Figs. 3 a′–e′ show the percentage effect instead of deviation index of the tumor. Deviation index fell with decrease in concentration of the drugs. Percentage effect ran parallel with the curve obtained from deviation index of tumor cells treated with MMC, CPA, CMA and 5 FU. In nitrogen mustard N-oxide treated cells, however, the deviation index increased regardless of decrease in concentration of the drug. These results indicated that the deviation index is related to death of cells in MMC, CPA, CMA and 5 FU.

On the other hand, the pH at high concentrations of drugs were variable as shown in Fig. 4, namely pH of 5 FU was alkaline and that of nitrogen mustard N-oxide was acid, while with less than 2×10^{-3} (4 μg of MMC, 200 μg of CPA, 500 μg of 5 FU and 1 μg of CMA in one ml) they were neutral. The following experiments were therefore performed at this concentration.



○—○ Mitomycin C; A=2 mg
△—△ Cyclophosphamide; A=100 mg
×—× Nitrogen mustard N-oxide; A=50 mg
●---● Chromomycin A_3; A=0.5 mg
□—□ 5-Fluorouracil; A=250 mg

FIG. 4. pH value in relation to dilution with P.B.S..

B) Comparison between in vitro and in vivo experiments

a) Tumor growth: Table 1 (a) shows the relationship between deviation index and 50% survival time. The second paragraph indicates the examined drugs in order of deviation index, which was about related with 50% survival time in MMC, 5 FU, CMA treated groups. CPA, however, was not always in

TABLE 1 (a). Comparison of Deviation Index of Tumor and 50 Percent Survival Time of Tumor Bearing Animals Treated with Drugs

Tumor	Drug	Tumor APD (D.I.)*	50% survival time (day)**	*P*-value
Ehrlich ascites tumor	MMC	+26.9±3.4	24.0 (19-30)	$P>0.01$
	5-FU	+14.6±2.5	21.4 (15-28)	$P>0.01$
	CPA	+2.4±2.1	16.4 (11-22)	$P<0.05$
	MTX	+0.4±1.8	15.2 (11-19)	$P<0.05$
	Vincristin	−3.0±2.0	15.3 (12-20)	$P<0.05$
	CMA	−8.6±2.0	14.9 (8-21)	$P<0.05$
	Control		14.1 (11-17)	
AH-130	MMC	+29.3±2.5	25.8 (19-34)	$P>0.01$
	5-FU	+20.9±3.1	21.1 (16-27)	$P>0.01$
	CPA	+2.0±2.8	13.7 (9-21)	$P<0.05$
	CMA	−3.3±2.2	13.2 (8-19)	$P<0.05$
	Control		11.9 (8-15)	
Yoshida sarcoma	MMC	+27.1±3.6	9.2 (7-13)	$P>0.01$
	5-FU	+1.8±2.6	8.5 (6-13)	$0.01>P>0.05$
	CMA	+0.1±2.2	7.8 (7- 9)	$0.01>P>0.05$
	CPA	−1.2±2.4	13.1 (12-15)	$P>0.01$
	Control		6.4 (5- 9)	
FM 3 A	MMC	+22.5±3.1	28.0 (24-35)	$P>0.01$
	5-FU	+10.1±3.1	27.4 (24-33)	$P>0.01$
	CMA	+8.9±3.0	22.2 (18-26)	$P<0.05$
	CPA	+2.3±2.1	20.0 (15-26)	$P<0.05$
	Control		20.6 (17-26)	
Friend virus induced tumor	5-FU	+18.9±2.0	21.0 (15-28)	$P>0.01$
	CMA	+16.8±2.1	22.5 (17-26)	$P>0.01$
	MMC	+2.5±2.6	15.9 (9-22)	$P<0.05$
	CPA	+1.3±2.2	17.6 (11-26)	$0.01>P>0.05$
	Control		13.3 (10-18)	

* D.I.±S.D., ** mean (range)

agreement with respect to deviation index and 50% survival time. Table 1 (b) shows a summary of Tab. 1 (a) as regards relationship between deviation index and survival ratio. Sensitivity of drugs against tumor was slightly different with each tumor.

The deviation indices obtained from solid forms of Ehrlich tumor and Yoshida sarcoma were compared with the tumor growth after treatment. As shown in Fig. 5, the results between the index of the tumor and the tumor growth were correlated in these groups treated with MMC, CMA, CPA, but was not correlated in Yoshida sarcoma treated with 5 FU.

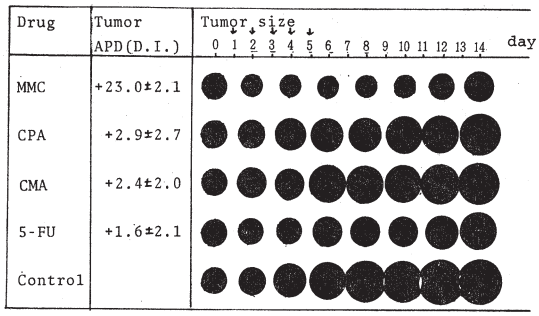
TABLE 1 (b). Tumor-Drug Relationship: Comparison of Deviation Index of Tumor and Survival Ratio of Tumor Bearing Animals Treated with Drugs

Drug→ / Tumor ↓	MMC		5-FU		CMA		CPA	
	D.I.*	Ratio**	D.I.*	Ratio**	D.I.*	Ratio**	D.I.*	Ratio**
AH-130	+29.3	2.17	+20.9	1.78	−3.3	1.11	+2.0	1.15
Ehrlich ascites tumor	+26.9	1.71	+14.6	1.52	−8.6	1.08	+2.4	1.16
Yoshida sarcoma	+27.1	1.44	+1.8	1.33	+0.1	1.22	−1.2	2.05
FM 3 A	+22.5	1.36	+10.1	1.33	+8.9	1.09	+2.3	1.00
Friend virus induced tumor	+2.5	1.19	+18.9	1.58	+16.8	1.69	+1.3	1.32

* mean of deviation index of tumor

** 50% survival time of tumor bearing animals treated with drugs divided by that of control

Solid form of Ehrlich carcinoma



Solid form of Yoshida sarcoma

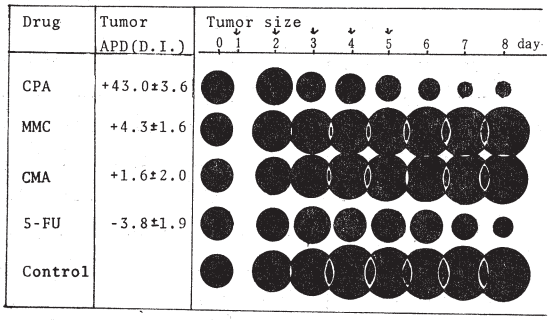


FIG. 5. Deviation index of solid Ehrlich carcinoma and Yoshida sarcoma and tumor size after treatment.

b) Side effects: To observe the toxicity of drugs, deviation index of liver and 50% survival time were investigated and shown in Table 2. The second paragraph indicates the order of deviation index of the liver. When the values of deviation indices were greater, the survivals were shorter.

TABLE 2. Difference in Liver APD and 50 Per cent Survival Time According to Strain of Mice

Drug	Strain of mouse	Liver APD (D.I.)*	50% survival time (day)**	*P*-value***
MMC	dd	+15.2±1.5	15.1 (11-22)	
	ICR Swiss	+0.9±1.4	30.0 (24-36)	$P>0.01$
	CBA	−4.6±1.8	31.0 (24-38)	$P>0.01$
	C 57 BL	−7.6±1.8	31.0 (27-37)	$P>0.01$
CPA	dd	+16.2±1.8	9.6 (6-14)	
	ICR Swiss	−2.5±1.8	16.9 (12-28)	$P>0.01$
	CBA	−3.2±2.4	12.6 (8-18)	$0.01>P>0.05$
	C 57 BL	−9.1±2.6	16.8 (10-22)	$P>0.01$
CMA	dd	+14.2±1.8	5.5 (3- 8)	
	ICR Swiss	+10.4±1.4	7.2 (4-11)	$P<0.05$
	C 57 BL	−0.6±1.4	10.8 (5-18)	$P>0.01$
	CBA	−0.7±1.4	10.9 (6-17)	$P>0.01$
5-FU	dd	−0.7±1.9	6.9 (5- 9)	
	C 57 BL	−2.4±2.2	6.6 (5- 8)	$P<0.05$
	ICR Swiss	−3.6±1.6	7.6 (4- 9)	$P<0.05$
	CBA	−5.4±1.9	8.0 (7-10)	$P<0.05$

* D.I., S.D., ** mean (range), *** value of dd strain as control

The side effects including survival time, loss of weight, leukocyte count and albuminuria, were investigated in various strains of mice treated with MMC. As shown in Table 3, the fifty percent survival time of mice was 4.5 days in dd, followed by C 3 H/He, CBA and C 57 BL; leukocyte count of the mice decreased most in dd, and slightest in C 57 BL; the presence of albuminuria showed no difference between them. The results of leukocyte count were well paralleled with the deviation indices of the liver of mice.

c) Host-tumor-drug relationship: The deviation index of the tumor was expressed as (T) and the index of the liver as (L). If the (T) value is greater, the agent must be more effective and if the (L) value is greater, the host will be killed by the effect of the agent because of decrease of host resistance. Therefore, a parameter calculating (T) − (L) was taken into consideration to estimate the effect of the drug. From the results in Table 4, the values were well paralleled with the fifty percent survival times.

TABLE 3. Host-Drug Relationship: Deviation Index of Liver to MMC and 50% Survival, Loss of Weight, Leukocyte Count and Albuminuria of Mice Treated with MMC in a Dose of 5 μg/g

Strain of mouse	Liver APD (D.I.)	50% survival time (day)**	Loss of weight		Leukocyte count			Albuminuria	
			before treatment	3 d. after t.	before t.**	1 d. ** after t.	3 d. ** after t.	before t.	3 d. after t.
dd	+15.2 ±1.5*	4.5 (3- 6)	21.4	17.9	12200 (17200 -7800)	10000 (17600 -5200)	2000 (3200 -1400)	0/10	+6/10
C3H/He	−4.4 ±1.6	6.2 (5- 8)	22.3	20.1	10500 (14400 -8400)	6000 (8800 -3100)	3700 (5200 -1200)	0/10	+6/10
CBA	−4.6 ±1.8	6.4 (4- 7)	23.2	20.6	10100 (17200 -7200)	3700 (5200 -2000)	3250 (4800 -900)	0/10	+5/10
C 57 BL	−7.2 ±1.4	8.3 (5-12)	22.5	22.2	9600 (15600 -5200)	3200 (6000 -1600)	3000 (6500 -1000)	0/10	+9/10

* D.I.±S.D., ** mean (range)

TABLE 4. Host-Tumor-Drug Relationship: Comparison Between (T)−(L) and 50 Percent Survival Time in Various Strains of Mice

Drug	Strain of mice	Tumor APD (D.I.) (T)	Liver APD (D.D.) (L)	(T)−(L)*	50% survival time minus control (day)**
MMC	C 57 BL	+26.9±3.4	−7.6±1.4	+34.5±4.8	13.0±2.2
	CBA		−4.6±1.8	+31.5±5.2	12.5±2.1
	ICR Swiss		+0.9±1.4	+26.0±4.8	10.9±1.9
	dd		+15.2±1.2	+10.7±4.7	4.4±1.7
5-FU	CBA	+14.8±2.5	−5.4±1.9	+20.2±4.4	9.0±2.2
	ICR Swiss		−3.6±1.6	+18.4±4.1	7.3±1.9
	C 57 BL		−2.4±2.0	+17.2±4.5	10.7±2.2
	dd		−0.7±1.9	+15.5±4.4	4.8±2.2
CPA	C 57 BL	+2.4±2.1	−9.1±2.6	+11.5±4.7	3.8±2.4
	CBA		−3.2±2.4	+5.6±4.5	2.9±1.8
	ICR Swiss		−2.5±1.8	+4.8±3.9	2.3±1.7
	dd		+16.2±1.8	−13.8±3.9	0.7±1.8
CMA	CBA	−8.9±2.0	−0.7±1.7	−8.2±3.7	3.1±2.1
	C 57 BL		−0.6±1.4	−8.3±3.4	4.3±2.3
	ICR Swiss		+10.4±1.4	−19.3±3.4	0.8±1.9
	dd		+14.2±1.8	−23.1±3.8	0.2±2.3

* D.I.±S.D., ** mean±S.D.

d) Clinical application: As shown in Table 5, in 17 patients treated with appropriate drugs, 6 cases with deviation index of more than +25 were closely correlated with the clinical response of drugs. However, the effect of CPA was not always in agreement with the deviation index of tumor. Moreover, as shown in Table 6, the side effects of MMC were likely to occur in patients

TABLE 5. Relationship between Index of Tumor and Clinical Response of Patients with Malignancy Treated with Appropriate Carcinostatic Agents (Inoperable)

Patient	Age Sex	Tumor APD (D.I.)		Total dose (mg)	Clinical response
1. Hodgkin's disease	20 M	MMC	−13.6	40	−
		CPA	+37.3	3300	−
		CMA	+5.5		
2. mixed tumor of salivary gland	59 M	MMC	−25.3	50	−
		CPA	−3.1	6000	−
		CMA	−43.5		
3. gastric cancer	32 F	MMC	+26.5	40	+
		CPA	−60.1		
		CMA	+32.1	5	−
4. gastric cancer	59 F	MMC	+38.2	40	++
		CPA	−6.7		
		CMA	−2.0		
5. gastric cancer	57 F	MMC	−7.4	30	−
		CPA	+10.4		
		CMA	−20.3		
6. gastric cancer	57 M	MMC	−7.4	32	−
		CPA	+10.4		
		CMA	−20.3		
7. myosarcoma of skin	59 F	MMC	+64.5	12	−
		CPA	+45.0		
		CMA	+27.2		
		5 FU	+71.6	5500	++
8. reticulosarcoma	23 M	MMC	−9.0		
		CPA	+16.0	3100	+
		CMA	−13.0		
		5 FU	+39.1	6000	++
9. colon cancer	54 F	MMC	−0.4	16	−
		CPA	−3.9		
		CMA	−16.5		
		5 FU	−12.1		
10. gastric cancer	64 M	MMC	+4.4	28	−
		CMA	+3.1		
		5 FU	−2.8		
11. reticulosarcoma	48 M	MMC	+9.1		
		CPA	+18.8	3500	+
		CMA	+17.4		
		5 FU	+16.9		
12. skin cancer	33 F	MMC	+3.3	40	−
		CMA	−23.3		
		5 FU	−6.3		
13. gastric cancer	72 M	MMC	+4.5		
		CMA	+8.4		
		5 FU	+38.9	5500	++

TABLE 5. (*Continued*)

Patient	Age Sex	Tumor APD (D.I.)		Total dose (mg)	Clinical response
14. gastric cancer	44 F	MMC	+1.3	40	−
		CMA	+1.3		
		5 FU	−27.0		
15. cancer of gall bladder	41 M	MMC	+17.4	40	−
		CMA	+12.0		
		5 FU	+17.4	5000	+
16. gastric cancer	48 M	MMC	+37.0	40	+
		CMA	+34.4		
		5 FU	+43.3	5000	++
17. gastric cancer	54 M	MMC	+13.6	40	−
		CMA	+11.3		
		5 FU	+0.4		

− no effect
+ decrease of tumor size to less than 25% in its diameter
++ decrease of tumor size to less than 50% in its diameter
+++ decrease of tumor size to more than 50% in its diameter and decrease of ascites volume

having a high value of deviation index of the liver; namely, leukopenia of less than 4,500 WBC count occurred in seven patients after MMC treatment whose deviation indices of liver to MMC were more than +10, except one case, No. 8. As shown in Table 7, except one case, No. 4, when the values of (T)−(L) were greater, patients with carcinoma of the stomach treated with curative radical surgery plus chemotherapy lived longer.

DISCUSSION

The majority of carcinostatic agents eventually lead the cells to death through some processes, and it is reasonable to judge the sensitivity of each drug by the degenerative preocesses in cells. Lysosomal enzymes are related to cell destruction, and of these acid phosphatase exists widely in normal and tumor cells, and its activity can be determined by a simple technique no matter how small it may be.

The effectiveness of a drug should be considered from the tumor and host sides, and, in the present study, it was judged by the index of both sides. Besides the question of the tumor side, the judgement of side effects *in vitro* remains unsolved. Some judge them by the depression of bone marrow cells[30)31)] and others by the suppression of reticuloendothelial cells[32)33)]. Liver is also a tissue utilized to predict the side effects of a drug and is used most frequently for studies of acid hydrolase that are bound to the lysosome[34)35)36)].

Acid phosphatase activity was measured in the tumor and liver. The effectiveness of the drug was determined by the ratio of the specific activity of tumor and liver cells treated with the agent to that of untreated tumor

TABLE 6. Relationship between Deviation Index of Liver and Side Effects on Patients with Carcinoma of the Stomach Treated with MMC

Patients			Total dose (mg)	Liver APD (D.I.)	Leukocyte count			Liver function			
No.	Yr.	Sex			Before	After 1 w.	treated 3 w.	s-GOT Before	After treated 3 w.	s-GPT Before	After treated 3 w.
1	61	M	20*	+2.8	7600	8200	6600	24	24	16	23
2	70	M	20*	**+22.9**	**6700**	4300	**2500**	10	20	7	18
3	56	M	26*	−1.4	9400	—	8600	12	25	11	19
4	44	M	20*	−7.0	11600	10700	8900	18	41	17	27
5	48	M	26*	−4.5	7200	7000	6200	19	59	16	45
6	73	M	20*	−3.3	7400	—	6300	10	27	4	17
7	64	M	20*	−6.0	6200	5800	5200	19	19	12	8
8	26	M	20*	**+18.3**	**6600**	8100	**6800**	16	28	18	18
9	54	F	22*	+1.2	5100	—	6400	16	12	8	9
10	45	M	20*	**+16.0**	**6600**	4600	**3400**	14	55	10	29
11	40	M	26*	−12.6	5300	—	5400	25	29	17	22
12	42	F	26*	+7.3	5300	7800	6200	15	17	10	14
13	48	M	40**	+1.1	7800	9800	6300	20	21	16	16
14	56	F	40**	**+14.1**	**6900**	7300	**4100**	18	15	8	20
15	68	M	40**	+4.2	7500	7700	5900	22	16	10	13
16	66	M	40**	+2.1	10300	16400	11000	32	35	16	18
17	59	M	40**	−4.3	7800	6200	6900	56	55	54	50
18	34	F	40**	**+15.6**	**9000**	8600	**4500**	22	36	14	24
19	43	M	50**	−2.1	7800	6800	6400	27	34	28	34
20	65	M	32**	+5.0	6200	4200	4800	14	17	9	10
21	62	M	40**	+0.8	7400	7200	8100	14	21	16	20
22	36	M	40**	**+27.9**	**7300**	4100	**2300**	17	24	15	30
23	34	F	40**	**+12.8**	**6100**	4500	**4000**	22	36	14	24
24	28	F	40**	+0.2	4500	11600	14300	40	66	19	37
25	52	F	40**	**+12.2**	**6300**	5500	**4000**	24	20	25	18

* to hepatic artery with one shot injection
** injection with 4 mg of MMC twice a week

and liver cells, and the value was expressed as the deviation index.

The deviation index of the liver may show algebraically the degree of host defence mechanism against tumor which has been already mentioned in "the limitations and adverse effects of cancer chemotherapy"[37]. Thus the deviation index of tumor (T) minus that of liver (L) may show synthetically the effectiveness of drug against individuals with cancer; When (T)−(L) value is great, the tumor bearing host will live longer, whereas if (T)−(L) is low adverse effects may occur because of the depression in host resistance[38].

The results showed that the sensitivity of the agents was different for each tumor and that a possibility of host resistance against the agent would differ in individual hosts as has already been reported in the biochemical

TABLE 7. Follow-Up Results of Patients with Carcinoma of the Stomach Treated with Surgery Plus Chemotherapy in Relation to the Deviation Indices of Tumor and Liver

Patients			Histologic diag.	Tumor APD(T)	Liver APD(L)	(T)−(L)	Total doses (mg)	Survival time after surg. and chemoth ***
No.	Yr.	Sex						
1	70	M	Ad.*	−10.2	**+22.9**	−33.1	20	4 M. death
2	54	F	Ca. S**	−24.2	+1.2	−25.4	22	6 M. death
3	48	M	Ad.	−1.9	−4.5	+2.6	26	7 M. survival
4	61	M	Ad.	+7.1	+2.8	+4.3	20	12 M. death
5	73	M	Ad.	+7.0	−3.3	+10.3	20	6 M. death
6	64	M	Ad.	+4.4	−6.0	+10.4	20	14 M. death
7	56	M	Ad.	+14.7	−1.4	+16.1	26	14 M. survival
8	43	M	Ad.	**+26.5**	−2.1	+28.6	50	27 M. survival
9	44	M	Ad.	**+31.1**	−7.0	+38.1	20	12 M. survival
10	65	M	Ad.	**+47.1**	+5.0	+42.1	30	24 M. death
11	48	M	Ad.	−31.5	+1.1	−32.6	40	7 M. death
12	66	M	Ad.	−9.6	+2.1	−11.7	40	5 M. death
13	56	F	Ad.	+8.2	**+14.1**	−5.9	40	5 M. death
14	26	M	Ca. S	**+27.1**	**+18.3**	+8.3	20	4 M. death
15	45	M	Ad.	**+30.0**	**+16.0**	+14.0	20	12 M. death
16	68	M	Ad.	**+25.4**	+4.2	+21.2	40	16 M. survival
17	59	M	Ad.	+1.2	−4.3	+5.5	40	10 M. death
18	34	F	Ca. S	**+32.8**	**+15.6**	+15.2	40	8 M. death
19	62	M	Ad.	**+47.5**	+0.8	+46.7	40	18 M. survival

* Ad.: Adenocarcinoma, ** Ca.S: Carcinoma simplex, *** on Oct. 31, 1970

studies of human tumors[39)40)]. Furthermore, the results of this sensitivity test on the judgement of tumor and hostal cells were well paralleled with the anticancer effectiveness in case of MMC, CMA and 5FU. However, this *in vitro* test was in disagreement with the *in vivo* results in case of CPA.

This different result with CPA may depend on the action of the drug, the rate of distribution and excretion from the body; CPA is inactive *in vitro* and is metabolized to an active form in the liver[41)], lung or kidney[42)] and then the active form is rapidly cleared from the body[43)], and it has been stated that sensitivity to CPA is cell cycle dependent[44)] and is determined by the proliferative state of a cell population[45)]. However, a high deviation index of solid tumor *in vitro* may indicate a possibility that CPA was activated by various factors except tumor cells, since the ascites form did not activate CPA; Activation of CPA may be due to the presence of interstitial tissue of solid tumor[46)].

Some difference in the sensitivity to 5FU may depend mainly on the short incubation period *in vitro*, because 5FU interferes competitively with several metabolic processes but most prominently, after its conversion to

nucleoside, with the enzyme of thymidine synthetase[47)~49)]. Again, the difference may also depend on a fraction of cells in the proliferative state[50)51)], sicne 5 FU is much less active on cells which are in a nonproliferative state[52)].

The wide sensitivity of MMC against the tumor cells *in vitro* may depend on direct cell damage since MMC is a short acting cytocidal drug, while the sensitivity may be modified in itself by an action of the agent as "lysosome labilizer". However, at the molecular level, it seems to cause a specific block in DNA synthesis and to have little effect on protein or RNA synthesis[53)~55)], whereas, at high levels of the agent, cellular RNA and protein synthesis are also suppressed[56)]. There have been recently some reports that sensitivity to MMC is to some extent, but not entirely, correlated with the abscence of "host cell reactivating enzymes"[57)58)] and their effect on cross-linked DNA and with the degree of DNA cross-linking[59)]. Accordingly, the relation of mitomycin-induced degeneration of DNA to the primary action of the antibiotic is not certain, but the phenomenon that MMC kills the cells directly or acts on the cells as lysosome labilizer will be a secondary one, since there is more than one metabolism responsible for sensitivity to MMC.

The weak sensitivity to CMA may depend on the short incubation time *in vitro* in this experiment, since the response to the drug is said to be characterized by late destruction of tumor cells[60)] and a specific inhibition of RNA, probably as an inhibition of nuclear RNA synthesis[61)]. In addition, CMA may directly inhibit the release and activation of lysosomal enzymes, especially acid phosphatase, in tumor cells.

CONCLUSION

Judging drugs having different actions at the same dose level and with the same incubation time *in vitro* still remains a problem to be solved, in any attempt to translate from model systems in design of human clinical trials[62)63)]. However, this method appears simple and worthwhile in the drug selection for cancer chemotherapy, and a short acting drug such as MMC will be clearly anticipated for the effect of drug on tumor as well as side effects by this method.

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REFERENCE

1) Yamazaki, S., Nitta, K., Hikiji, T., Nogi, M., Takeuchi, T.. Yamamoto, T. and Umezawa, H., Cylinder plate method of testing the anti-cell effect: Studies on antitumor substances produced by actinomycetes, XII., *J. Antibiot.* (*Tokyo*), **9**, 135, 1956.
2) Yamamoto, T., Komeiji. T., Nishioka, K., Takeuchi, T. and Nitta, K., Cell-agar-plate (CAP) method for assaying antitumor activity and its application to the elucidation of the mode of action, *Gann*, **47**, 424, 1956 (in Japanese).
3) Miyamura, S., A determination method for anticancer action of antibiotics by agar plate diffusion technique, *Antibiot. Chemother.*, **6**, 280, 1956.
4) Miyamura, S. and Niwayama, S., An agar plate diffusion method using Hela cells for antitumor screening, *Antibiot. Chemother.*, **9**, 497, 1959.
5) Siminoff, P. and Hursky, V. S., Determination of mammalian cell (strain Hela) inhibition by an agar diffusion technique, I. Quantitative assay methods, *Cancer Res.*, **20**, 615, 1960.
6) DiPaolo, J. A., *In vitro* test systems for cancer chemotherapy, I. Inhibition of dehydrogenases and growth in the Ehrlich ascites tumor, *Cancer Res.*, **23**, 183, 1963.
7) Knock, F. E., Sensitivity test for cancer chemotherapy, *Arch. Surg.* (*Chicago*), **91**, 376, 1965.
8) Eagle, H. and Foley, G. E., Cytoxicity in human cell cultures as a primary screen for the detection of antitumor agents, *Cancer Res.*, **18**, 1017, 1958.
9) Toplin, I., A tissue culture cytotoxicity test for large-scale cancer chemotherapy screening, *Cancer Res.*, **19**, 959, 1959.
10) Wright, J. C., Cobb, J. P., Gumport, S. L., Safadi, D., Walker, D. G. and Golomb, F. M., Further investigation of the relation between the clinical and tissue culture response to chemotherapeutic agents on human cancer, *Cancer*, **15**, 284, 1962.
11) Hurley, T. D. and Yount, L. J., Selection of anticancer drug for palliation using tissue culture sensitivity studies, *Amer. J. Surg.*, **109**, 39, 1965.
12) Kondo, T., Imamura, T. and Ichihashi, H., *In vitro* test for sensitivity of tumor to carcinostatic agents, *Gann*, **57**, 115, 1966.
13) Schrek, R., A method for screening chemotherapeutic agents against normal and leukemic cells of man, *Proc. Am. Assoc. Cancer Res.*, **3**, 148, 1960.
14) McDonald, G. O., Stroud, A. N., Brues, A. M. and Cole, W. H., *In vitro* and *in vivo* assay for drug effect on cancer cells, *Ann. Surg.*, **157**, 785, 1963.
15) Bruce, W. R., Meeker, Q. E. and Valeriote, F. A., Comparison of the sensitivity of normal hematopoietic and transplanted lymphoma colony-forming cells to chemotherapeutic agents administered *in vivo*, *J. Nat. Cancer Inst.*, **37**, 233, 1966.
16) Skipper, H. E. and Perry, S., Kinetics of normal and leukemic leukocyte populations and relevance to chemotherapy, *Cancer Res.*, **30**, 1883, 1970.
17) DeDuve, C., The lysosome, *Sci. Amer.*, **208**, 64, 1963.
18) Novikoff, A. B., Essner, E. and Quintana, N., Golgi apparatus and lysosomes, *Fed. Proc.*, **23**, 1010, 1964.
19) Weissmann, G., Lysosomes, *New Eng. J. Med.*, **273**, 1084, 1965.
20) DeDuve, C. and Wattiaux, R., Functions of lysosomes, *Ann. Rev. Physiol.*, **28**, 435, 1966.
21) Branders, D., Sloan, K. W., Anton, E. and Bloedorn, F., The effect of X-irradiation on the lysosomes of mouse mammary gland carcinomas, *Cancer Res.*, **27**, 731, 1967.
22) Anton, E. and Branders, D., Lysosomes in mice mammary tumors treated with cyclophosphamide. (Distribution related to course of disease), *Cancer*, **21**, 483, 1968.
23) Allison, A., The role of lysosomes in the action of drugs and hormones, *Advans. Chemother.*, **3**, 253, 1968.
24) Lowry, O. H., Roberts, N. R., Wu, M. L., Hixon, W. S. and Crawford, E. J., The

quantitative histochemistory of brain, II. Enzyme measurements, *J. Biol. Chem.*, **207**, 19, 1954.

25) Torriani, A., Influence of inorganic phosphate in the formation of acid phosphatase by Esherichia Coli, *Biochim. Biophys. Acta*, **38**, 460, 1960.

26) Chersi, A., Bernardi, A. and Bernardi, G., Studies on acid hydrolases, II. Isolation and properties of spleen acid phosphomonoesterase, *Biochim. Biophys. Acta*, **129**, 12, 1966.

27) Dipietro, D. L. and Sausan Zengerle, F., Separation and properties of three acid phosphatases from human placenta, *J. Biol. Chem.*, **245**, 3391, 1967.

28) Lowry, O. H., Rosebrough, N. J., Lewis Farr, A. and Randall, R. J., Protein measurement with the Folin phenol reagent, *J. Biol. Chem.*, **193**, 265, 1951.

29) Schrok, R., Biologic assay of adrenal cortex hormones by the method of unstained cell counts, *Proc. Soc. Exp. Biol. Med.*, **77**, 709, 1951.

30) Romsdahl, M. M., Malmgren, R. A., Valaitis, J., McGrath, R. G. and McGrew, A. E., The "response" of tumor cells and hematopoietic elements of the blood to cancer chemotherapy, *Surg.*, **59**, 584, 1966.

31) Frei, E. 3d., Symposium on immunosuppressive drugs, Effect of cancer chemotherapeutic agents on normal tissues in man, *Fed. Proc.*, **26**, 918, 1967.

32) Kondo, T. and Ohkubo, K., *In vitro* test for prediction of side effects of carcinostatic agents, *Gann*, **58**, 349, 1967.

33) Edwards, A. J. and Rowland, G. F., Relative effects of varying concentrations of cytotoxic drugs *in vitro* on tumor and reticuloendothelial tissue, *Brit. J. Surg.*, **55**, 687, 1968.

34) Gahan, P. B., Reversible activation of lysosome in rat liver, *J. Histochem. Cytochem.*, **13**, 334, 1965.

35) Schersten, T., Wahlqvist, L. and Johansson, L. G., Lysosomal enzyme activity in liver tissue from patients with renal carcinoma, *Cancer*, **23**, 608, 1969.

36) Kampshmidt, R. F. and Wells, D., Acid hydrolase activity during the induction and transplantation of hepatomas in the rat, *Cancer Res.*, **29**, 1028, 1969.

37) Kondo, T. and Moore, G. E., The limitations and adverse effects of cancer chemotherapy, *Surg. Form*, **9**, 607, 1959.

38) 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.

39) Bickis, I. J. and Henderson, I. W. D., Biochemical studies of human tumors, I. Estimation of tumor malignancy from metabolic measurements *in vitro*, *Cancer*, **19**, 89, 1966.

40) Bickis, I. J., Henderson, I. W. D. and Quastel, J. H., Biochemical studies of human tumors, II. *In vitro* estimation of individual tumor sensitivity to anticancer agents, *Cancer*, **19**, 103, 1966.

41) Foley, G. E., Friedman, O. M. and Drolet, B. P., Studies on the mechanism of action of cytoxan: Evidence of activation *in vivo* and *in vitro*, *Cancer Res.*, **21**, 57, 1961.

42) Brock, N. and Hohorest, H. J., Über die Aktivierung von Cyclophosphamid in Warmblüterorganismus, *Naturwissenshaften*, **49**, 611, 1962.

43) Kline, I., Gang, M., Tyrer, D. D. and Mantel, N., Duration of drug levels in mice as indicated by residual antileukemic efficacy, *Chemotherapy* (*Basal*), **13**, 28, 1968.

44) DeWys, W. D. and Kight, N., Kinetics of cyclophosphamide damage-Sublethal damage repair and cell-cycle-related sensitivity[1,2], *J. Nat. Cancer Inst.*, **42**, 155, 1969.

45) DeWys, W. D., Goldin, A. and Mantel, N., Hematopoietic recovery after large doses of cyclophosphamide: Correlation of proliferative state with sensitivity[1], *Cancer Res.*, **30**, 1962, 1970.

46) Kondo, T. and Muragishi, H., Mechanism of cyclophosphamide activation, *Gann*, **61**, 145, 1970.

47) Heidelberger, C. and Ausfield, F. T., Experimental and clinical use of fluorinated pyrimidines in cancer chemotherapy, *Cancer Res.*, **23**, 1226, 1963.
48) Heidelberger, C., Chemical carcinogenesis, chemotherapy: Cancer's continuing core challenges-G.H.A. clows memorial lecture[1], *Cancer Res.*, **30**, 1549, 1970.
49) Kent, R. J., Khwaja, T. A. and Heidelberger, C., Fluorinated pyrimidines: XXXIV. Structure-activity studies of methylated 5-Fluoro-2-deoxyuridine derivatives, *J. Med. Chem.*, **13**, 79, 1970.
50) Madoc-Jones, H. and Bruce, W. R., Sensitivity of L-cells in exponential and stationary phase to 5-Fluorouracil, *Nature* (*London*), **215**, 302, 1967.
51) Bruce, W. R. and Meeker, B. E., Comparison of the sensitivity of hematopoietic colony-forming cells in different proliferative state to 5-Fluorouracil, *J. Nat. Cancer Inst.*, **38**, 401, 1967.
52) Kenis, Y., Dose schedules and modes of administration of chemotherapeutic agents in man, *in* Scientific basis of cancer chemotherapy edited by Georges Mathe (Recent Results in Cancer Research, 21), Press. Spinger-Verlag Berlin. Heidelberg. New York, 1969.
53) Sekiguchi, M. and Takagi, Y., Effect of mitomycin C on the synthesis of bacterial and viral deoxyribonucleic acid, *Biochim. Biophys. Acta*, **41**, 484, 1960.
54) Schwartz, H. S., Sternberg, S. S. and Philips, F. S., Pharmacology of mitomycin C, IV. Effects *in vivo* on nucleic acid synthesis; Comparison with actinomycin D, *Cancer Res.*, **23**, 1125, 1963.
55) Szybalski, W. and Iyer, V. N., Crosslinking of DNA by enzymatically on chemically activated mitomycins and porfiromycins, bifunctionally "alkylating" antibiotics, *Fed. Proc.*, **23**, 946, 1964.
56) Carter, S. K., Mitomycin C (NSC-26980)—clinical brochure[1,2,3], *Cancer Chemother. Rep.*, **1**, 99, 1968.
57) Setlow, R. B. and Carrier, W. L., The disappearance of thymine dimers from DNA: An error-correcting mechanism, *Proc. Natl. Acal. Sci. U. S.*, **51**, 226, 1964.
58) Boyce, R. P. and Howard-Flanders, P., Release of ultraviolet light-induced thymine dimers from DNA in E. Coli K-12, *Proc. Natl. Acad. Sci. U. S.*, **51**, 293, 1964.
59) Terawaki, A. and Greenberg, J., Post-treatment breakage or mitomycin C induced cross-links in deoxyribonucleic acid of Esherichia Coli, *Biochim. Biophys. Acta*, **119**, 540, 1966.
60) Kaziwara, K., Watanabe, J., Komeda, T. and Usui, T., Further observations on the inhibiting effects of chromomycin A_3 on transplantable tumors, *Cancer Chemother. Rep.*, **13**, 99, 1961.
61) Yano, M., Kusakari, T. and Miura, Y., Intracellular transfer of nucleic acids, III. Fate of nucleic acids in rat ascites hepatoma cells, *J. Biochem.* (*Tokyo*), **53**, 461, 1963.
62) Freireich, E. J., Gehan, E. A., Rall, D. P., Schmidt, L. H. and Skipper, H. E., Quantitative comparison of toxicity of anticancer agents in mouse, rat, hamster, dog, monkey and man, *Cancer Chemother. Rep.*, **50**, 219, 1966.
63) Skipper, H. E., Schabel, F. M., Bruce Mellet, L., Montgomery, J. A., Wilkoff, L. J., Lloyd, H. H. and Brockman, R. W., Implications of biochemical cytokinetic, pharmacologic and toxicologic relationships in the design of optimal therapeutic schedules, *Cancer Chemother. Rep.*, **54**, 431, 1970.