Nagoya J. med. Sci. 33: 247-256, 1970

COLORIMETRIC ESTIMATION OF SUCCINIC DEHYDROGENASE ACTIVITY BY NEOTETRAZOLIUM CHLORIDE AS A TUMOR SENSITIVITY TEST TO CHEMOTHERAPEUTIC AGENTS

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ABSTRACT

In the search for tumor sensitivity test to chemotherapeutic agents succinic dehydrogenase activity by neotetrazolium chloride was measured colorimetrically.

The rate of formazan production was proportional to the amount of Ehrlich ascites tumor cells, accelerated by the addition of ascorbic acid, and inhibited by the addition of iodoacetic acid.

The inhibitory rate of formazan production of tumor cells treated with chemotherapeutic agents was well correlated with the survival time of mice. Of the chemotherapeutic agents, mitomycin C was the most effective, either singly or in combination with other drugs.

Considering the mechanisms of action of drug and of the host's defense, an *in vivo* tumor system was studied as a screening test for the chemotherapeutic agents.

INTRODUCTION

A simple, rapid and prospective test of tumor sensitivity to chemotherapeutic agents would be of great value in cancer chemotherapy.

Previous attempts to select chemotherapeutic agents by determining their effects *in vitro* have been made by us in 1966[1]. The principle of the test is to incubate a cell suspension with triphenyl tetrazolium chloride, which is reduced in the presence of active dehydrogenase enzyme systems to red colored formazan.

The test may be useful in predicting the tumoricidal effect of certain drugs, though drugs like cyclophosphamide would be missed by such *in vitro* screening tests on account of its low toxicity *in vitro*.

In the present study, the other tetrazolium salt, neotetrazolium chloride [2, 2′, 5, 5-tetraphenyl-3, 3′ (ρ-diphenylene)-dietrazolium chloride] (Sigma), was estimated for its succinic dehydrogenase activity. In addition, using this test a method was designed to find whether any of these systems could replace an

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Received for publication September 25, 1969.

in vivo tumor system as a screening test for chemotherapeutic agents.

MATERIALS AND METHODS

For the examinations Ehrlich ascites tumor cells were used. The cells were taken from mice inoculated intraperitoneally 7 days before.

Unless otherwise mentioned, the incubating medium was as follows; 0.5 ml of cell suspension (5×10^6 cells), 0.5 ml of 0.1 M sodium succinate, and 0.5 ml of 0.5% neotetrazolium chloride (NT) solution. Incubation was conducted in centrifuge tubes for 1 hour at 37°C in an incubator, and the tubes were gently shaken once during the period. The reaction was stopped by the addition of 0.5 ml of 25% trichloroacetic acid. After shaking, the violet colored formazan was extracted with 4 ml of acetone. After shaking and centrifugation, absorbance of the extracts was measured by a Hitachi-Perkin-Elmer UV-VIS spectrophotometer at 535 mμ.

All reagents were dissolved in pH 7.2 phoshate buffered saline prepared by mixing 100 ml of saline and 100 ml of a buffer consisting of 23.9 ml of 0.15 M KH_2PO_4 and 76.1 ml of 0.15 M Na_2HPO_4. NT was not completely soluble in buffered saline, so the insoluble granules were filtered and discarded.

RESULTS

In Vitro Tests

1. The rate of formazan production increased linearly with counts of cells, ranging 125–2000 $\times 10^4$ in number. With more than 2000×10^4 cells the formazan

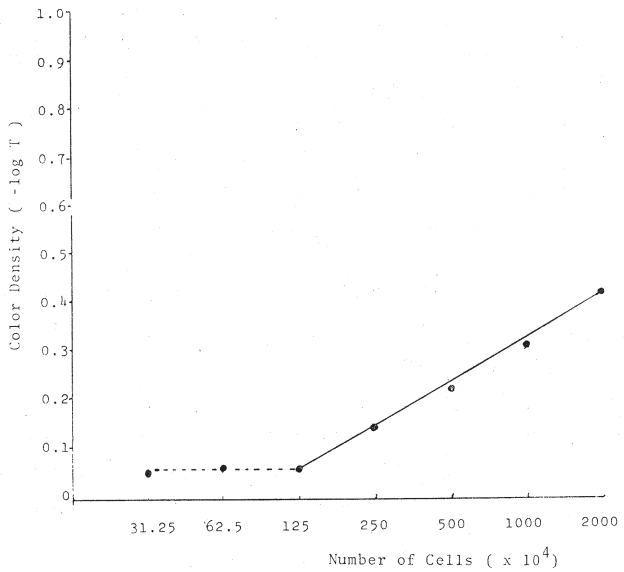

FIG. 1. Formazan production with NT in relation to number of cells.

was difficult to extract completely with acetone and with less than 125×10^4 cells the amount was minimum and not measurable, as shown in Fig. 1.

2. The rate of reduction was direct up to 5 mg concentration of ascorbic acid with or without addition of cells, as shown in Fig. 2.

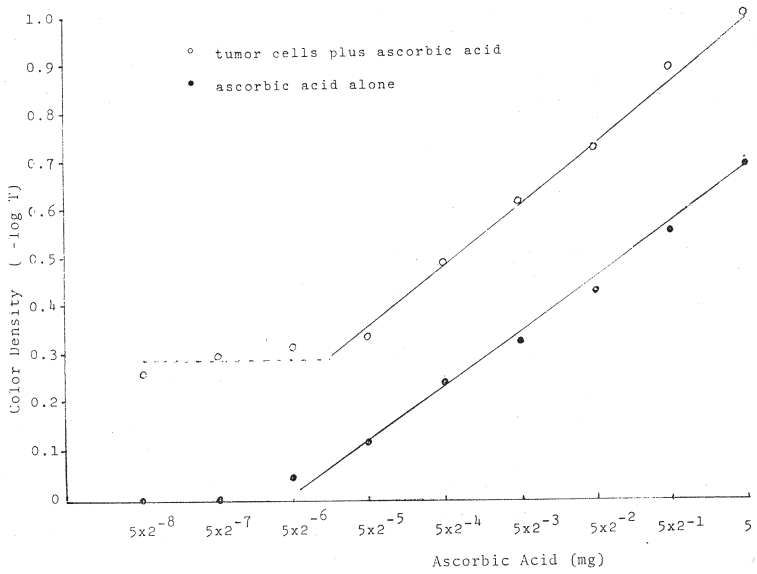

FIG. 2. Formazan production with NT in relation to concentration of ascorbic acid.

TABLE 1. Reduction of NT by Ehrlich Ascites Tumor Cells in the Presence of Iodoacetic Acid

CH_2ICOOH (Mol)	Absorbance ($-\log T$)
0.1	0.000
0.1×2^{-1}	0.055
0.1×2^{-2}	0.215
0.1×2^{-3}	0.265
0.1×2^{-4}	0.240
0.1×2^{-5}	0.215
0.1×2^{-6}	0.236
Control	0.207
	0.212
	0.220

3. For studying the enzyme system which may be responsible for the formazan production, iodoacetic acid was tested as an enzyme inhibitor. Iodoacetic acid was dissolved in pH 7.2 phosphate buffered saline and was incorporated into the incubating medium. As shown in Table 1, iodoacetic acid at 0.1 M inhibited totally formazan production. At 0.1×2^{-1} M, about 75%

inhibition was observed, and at less than 0.1×2^{-2} M, no inhibition was observed.

4. The other tetrazolium salt, triphenyl tetrazolium chloride (TTC) was next tested.

The procedure was the same as described in "MATERIALS AND METHODS" except that 0.5% TTC solution was used instead of 0.5% NT. The red colored

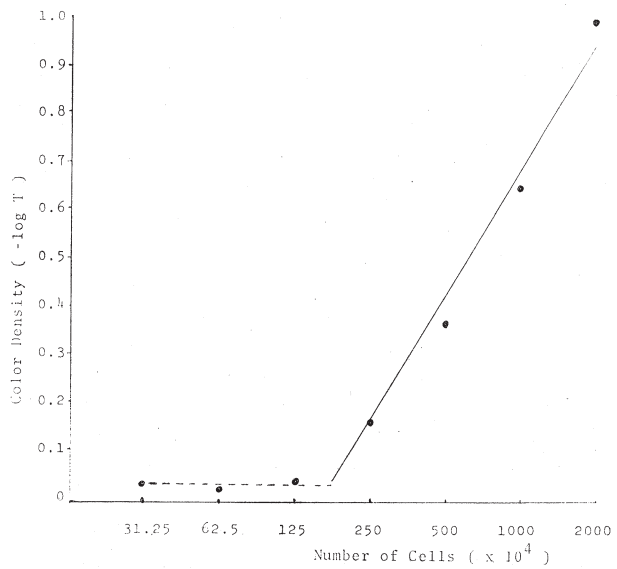

FIG. 3. Formazan production with TTC in relation to number of cells.

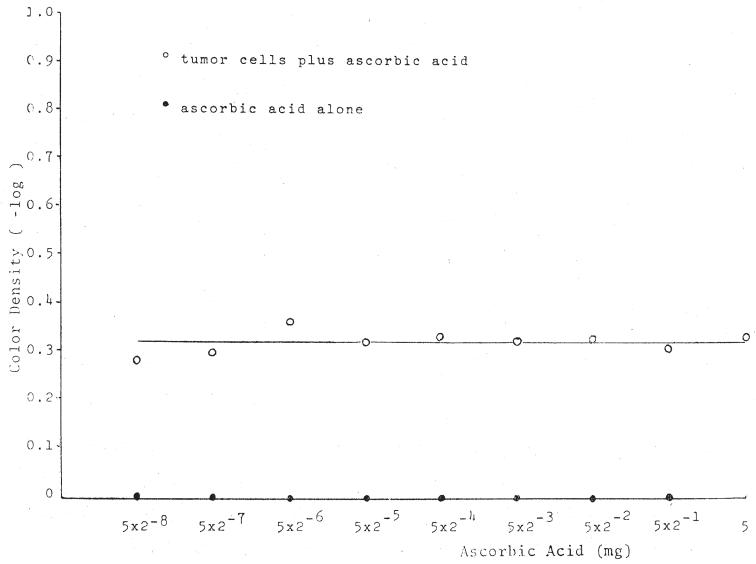

FIG. 4. Formazan production with TTC in relation to concentration of ascorbic acid.

formazan was measured by a spectrophotometer at 480 mμ. As shown in Fig. 3, formazan production was measurable with more than 125×10^4 cells, and increased with increase in the number of cells.

Addition of ascorbic acid into the incubating medium did not influence the formazan production, as shown in Fig. 4.

Iodoacetic acid at 0.1 and 0.1×2^{-1} M inhibited the formazan production, as indicated in Table 2.

TABLE 2. Reduction of TTC by Ehrlich Ascites Tumor Cells in the Presence of Iodoacetic Acid

CH_2ICOOH (Mol)	Absorbance ($-\log T$)
0.1	0.070
0.1×2^{-1}	0.145
0.1×2^{-2}	0.400
0.1×2^{-3}	0.460
0.1×2^{-4}	0.470
0.1×2^{-5}	0.450
Control	0.290
	0.310
	0.310

In Vivo Sensitivity Tests

1. SMA male mice were inoculated intraperitoneally with 3×10^6 viable cells as a 0.1 ml suspension. When ascites developed, they were divided into three experimental groups of 9 mice each: group 1 received drugs on the 7th day after tumor inoculation; group 2 on the 10th day; and group 3 on the 13th day. These experimental groups of mice were further divided into three subgroups of 3 mice each: of mitomycin C (MMC) treated; 5-fluorouracil (5-FU) treated; and cyclophosphamide (CPA) treated subgroups.

MMC, 5-FU and CPA were given by intraperitoneal injection and 0.05 ml of ascites was aspirated by a micro-syringe after certain intervals. Ascites were collected at 2, 6, 12, 20 and 40 hours after 1/2 LD_{50}* of drug administration in group 1. In group 2, they were collected at 2 and 4 hours, and on 1, 2, 3, and 5 days after 1/2 LD_{50} of drug administration. In group 3, they were collected at 2 and 4 hours, and on 1 and 2 days after LD_{50} of drug administration. Ascites thus obtained were poured into the incubating medium.

The results obtained by this procedure and the survival days of each mouse are shown in Table 3. Formazan production was the greatest with CPA, less with 5-FU, and the least with MMC in all three experiments. This indicates

* LD_{50}MMC, CPA, 5-FU, CM are 5.2 mg/kg, 432 mg/kg, 156 mg/kg, 2.12 mg/kg, respectively.

TABLE 3. Reduction of NT by Ehrlich Ascites Tumor Cells from Mice Treated with Chemotherapeutic Agents, after Various Periods

Inoculated after	Drug	Nr. mice	Formazan after administration of drugs (absorbance)							Survival day
			before	2 hrs.	6 hrs.	12 hrs.	20 hrs.	40 hrs.		
7 day	MMC	1	0.500	0.088	0.280	0.322	0.420	0.215		
		2	0.370	0.095	0.125	0.135	0.220	0.170		
		3	0.410	0.100	0.215	0.220	0.210	0.150		
		mean	0.426	0.094	0.207	0.226	0.283	0.178		
	5-FU	1	0.460	0.205	0.260	0.520	0.500	0.450		
		2	0.570	0.355	0.370	0.335	0.300	0.300		
		3	0.400	0.310	0.300	0.325	0.295	0.310		
		mean	0.477	0.290	0.310	0.393	0.365	0.353		
	CPA	1	0.470	0.335	0.300	0.412	0.490	0.555		
		2	0.490	0.400	0.400	0.398	0.400	0.405		
		3	0.510	0.310	0.405	0.380	0.380	0.350		
		mean	0.490	0.348	0.368	0.397	0.423	0.437		
10 days	MMC		before	2 hrs.	4 hrs.	1 days	2 days	3 days	5 days	
		1	0.560	0.350	0.350	0.262	0.040	0.150	0.175	25
		2	0.410	0.085	0.420	0.340	0.330	0.450	0.215	30
		3	0.390	0.160	0.160	0.132	0.100	0.226	0.165	37
		mean	0.453	0.198	0.310	0.245	0.157	0.275	0.185	30.7
	5-FU	1	0.300	0.180	0.320	0.363	0.350	0.310	0.260	20
		2	0.470	0.340	0.370	0.490	0.435	0.505	0.370	22
		3	0.630	0.460	0.440	0.500	0.480	0.370	0.285	19
		mean	0.467	0.327	0.380	0.451	0.422	0.395	0.305	20.3
	CPA	1	0.550	0.250	0.270	0.530	0.565	0.390	0.365	24
		2	0.500	0.300	0.442	0.620	0.350	0.385	0.360	20
		3	0.400	0.300	0.230	0.520	0.385	0.500	0.360	30
		mean	0.483	0.283	0.314	0.557	0.433	0.425	0.362	24.7
13 days	MMC		before	2 hrs.	4 hrs.	1 day	2 days			
		1	0.560	0.462	0.400	0.380	0.275			21
		2	0.495	0.340	0.335	0.290	0.278			24
		3	0.500	0.415	0.240	0.362	0.323			17
		mean	0.518	0.406	0.325	0.344	0.292			20.7
	5-FU	1	0.560	0.458	0.315	0.360	0.320			22
		2	0.510	0.432	0.325	0.290	0.400			18
		3	0.515	0.455	0.415	0.445	0.435			20
		mean	0.528	0.448	0.352	0.365	0.385			20.0
	CPA	1	0.550	0.435	0.390	0.385	died			15
		2	0.480	0.330	0.320	0.330	0.342			22
		3	0.530	0.435	0.400	0.500	0.510			18
		mean	0.537	0.400	0.370	0.405	0.426			18.3

that MMC is the most effective electron inhibitor among three drugs. Between CPA and 5-FU, the difference was negligible.

After the injection of chemotherapeutic agents, the quantity of formazan was reduced to a minimum after 2 hours in groups 1 and 2. The decrease was temporary and a gradual increase followed. One day later, formazan production began to decrease, and the subgroups treated with MMC showed a decrease faster than the other two subgroups treated with either 5-FU or CPA. In accordance with the low grade of formazan production, the subgroups treated with MMC survived longer. In group 3, formazan production decreased slightly after the injection of these drugs. No or slight change in formazan production was observed in all three subgroups.

2. SMA male mice were inoculated intraperitoneally with 4×10^6 viable cells. Ten days later when ascites developed they were divided into five groups of 5 mice each: group 1 surved as untreated control; group 2 received 1/4 LD_{50}* chromomycin A_3 (CM) combined with 1/4 LD_{50} MMC; group 3 1/4 LD_{50} MMC plus 1/4 LD_{50} 5-FU; group 4 1/4 LD_{50} MMC plus 1/4 LD_{50} CPA; and group 5 1/4 LD_{50} 5-FU plus 1/4 LD_{50} CPA. All these drugs were given intraperitoneally.

The formazan production was measured on the 1, 2, 4, 6, 8, and 12th day after the drug administration. As shown in Fig. 5, the CM plus MMC treated group showed the lowest formazan production throughout, among these groups. The 3 other treated groups showed values in between the group treated with

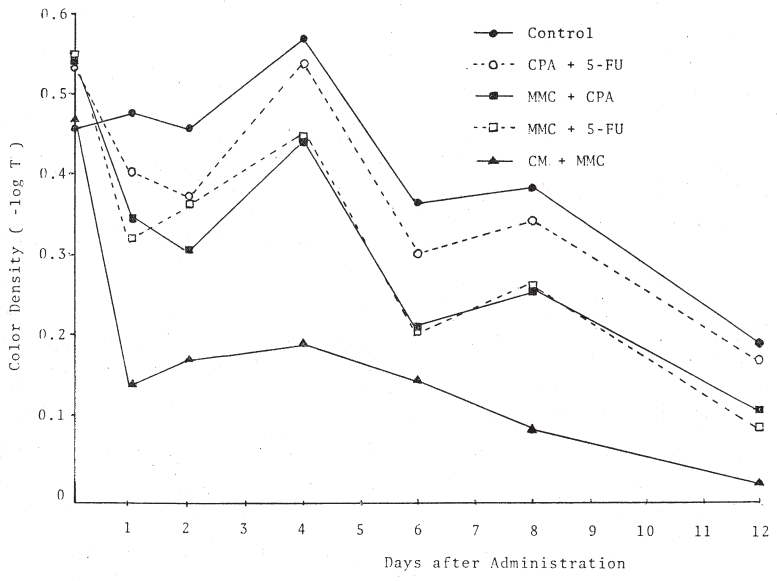

FIG. 5. Formazan production with NT after administration of chemotherapeutic agents. Each point represents the mean values of 5 determinations.

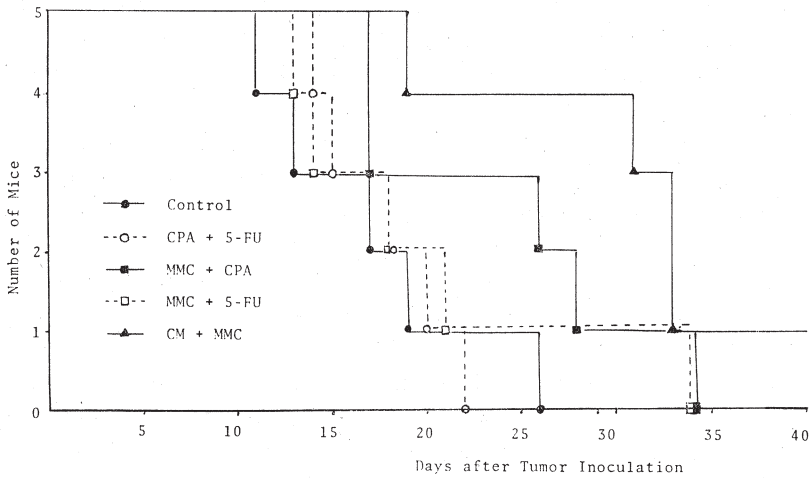

FIG. 6. Survival days of mice treated with chemotherapeutic agents.

CM plus MMC and the untreated control group. The reason why the values were rather low in the terminal days may be due to the cells becoming diluted by the marked development of ascites around the days. Much higher values may be obtained if the formazan production is measured by per cell instead of ascitic volume. These figures were well correlated with their survival times as indicated in Fig. 6, in which the mice treated with CM plus MMC survived longest.

DISCUSSION

Based on the observations by Strauss *et al.*[2] that tumor tissue would reduce the TTC more readily than normal tissue, and by Dallner[3] that the dehydrogenase functional system is produced only by living cells and rapidly inactivated when cell death occurred, the tetrazolium salts were used for drug sensitivity tests.

Using NT, as well as TTC, formazan production by ascites tumor cells was found to be proportional to the amount of cells, and this method is, therefore, applicable in the measurement of enzyme activity of living cells.

It is particularly interesting that the reduction of NT was dependent on ascorbic acid, though TTC was independent of the method used. Therefore, TTC would be more suitable than NT for the measurement of succinic dehydrogenase activity itself.

Application of dehydrogenase activity in *in vitro* drug sensitivity test has been proposed by many authors[1)4)5)6)7)8)9)]. However, there exists some disagreement between *in vitro* and *in vivo* drug sensitivities. Such disagreement may be due to differences in the mechanism of individual host's defense. The

biological effects of chemotherapeutic agents are widely different; alkylating agents and mitomycin C react directly with DNA and prevent its continued synthesis, actinomycin D and chromomycin A_3 inhibit RNA synthesis, antimetabolites such as 5-fluorouracil prevent the formation of nucleotides and DNA, some plants extracts affect protein synthesis, and steroids have a rather obscure action on proteins and ribosomal RNA[10]. As the *in vitro* tests may be particularly useful only when a drug is brought into direct contact with tumor cells, they would not be of value in case of the long acting drugs such as antimetabolites and of drugs like cyclophosphamide which showing efficacy after undergoing structural changes *in vivo*, and when host defense mechanisms are considered.

For this reason, in a present study a screening model was designed to approach as near as possible an *in vivo* situation. MMC as a cytocidal drug, 5-FU as an antimetabolite, and CPA as an alkylating agent were administered intraperitoneally. The results revealed that MMC showed faster efficacy than the other two drugs, though the patterns were almost the same.

The aim of simultaneous administration of two or more agents which differ in mechanisms of action, is to produce much greater effect than the individual compound without increasing the toxicity. In the study of individual drug sensitivity, MMC showed much more inhibitory effect on succinic dehydrogenase activity than the other two tested drugs. Also, in the study of combined drug sensitivity, when one of the other two drugs was administered simultaneously with MMC it showed more inhibitory effect than a combination of themselves.

The decrease in succinic dehydrogenase activity caused by dilution with the developing ascites remains a problem that requires to be solved in this procedure.

ACKNOWLEDGMENT

The authors are grateful for the criticisms of Prof. Shin Hoshikawa.

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