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161. Morizo Ishidate, Yoshio Sakurai, Hiroshi Imamura, and Ayako Moriwaki : Studies on Carcinostatic Substances. XXII.*2 Screening Method for Antimitotic Substance using the *in vitro*-Cultured Yoshida Sarcoma Cells.

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The experiment was reported in the preceding paper[1] on a convenient technique of culturing the Yoshida sarcoma cells in a test tube using a simple nutrient medium. By using this method, a new system of screening substances having antitumor activity could be established, which is very simple in procedure and believed to be widely applicable.

Of course many works have been published by many researchers on the same problem. In most of the instances, however, decrease of growth rate of the tumor cells affected by test compound was checked as an index of the effect.

In the present procedure, the usual cytomorphological determination was adopted, because the same technique and standard of judgement have been employed in this laboratory for many years in order to screen test compounds with animals bearing the Yoshida sarcoma. The determination was proved by these experiments to be nearly sensitive as that by growth-rate depression and also to have the advantage of discriminating true antimitotic effect from the acute general toxicity. Effect of test compound on the mitotic rate of cultured cells was observed at the same time through the period of incubation.

It was found that some of the compounds caused a direct damage or cytolysis of tumor cells within a short period after its addition into the culture medium. This direct damaging action is termed "primary effect," but this is regarded as not a promising factor in an antitumor activity of the compound, although the growth rate of the cell *in vitro* happened to fall even by such an acute and nonspecific toxicity.

The true antimitotic compound showed only a slight "primary effect" at an appropriate concentration, and after 48~72 hours' incubation, it usually induced a characteristic morphological aberration of nuclei in a large portion of the cell population, which lasted long and resulted in complete degeneration of the cells. Such a delayed and lasting effect is termed "secondary effect."

A minimum effective concentrations (MEC) of the various antitumor substances have been determined, at which a minimum observable "secondary effect" could be found.

It was believed that the present procedure could be used not only for a first-step screening of antitumor substances but as a method of analyzing their biological activity in any arbitrary condition.

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Experimental

Materials and Methods—Horse serum (HS), Earle's balanced saline solution (EBS), physiological saline solution (PS), K-penicillin G solution (P-soln.), and dihydrostreptomycin sulfate solution (S-soln.) were prepared as described in the preceding paper.[1]

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*2 Part XXI. : This Bulletin, **7**, 867(1959).

1) M. Ishidate, *et al.* : This Bulletin, **7**, 690(1959).

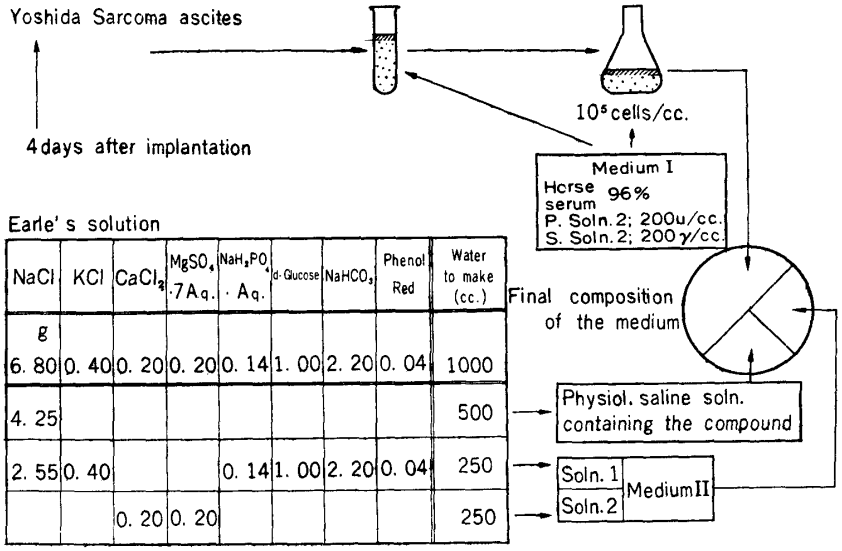
Solution-1 (S-1): NaCl 2.55 g., KCl 0.40 g., $NaH_2PO_4 \cdot H_2O$ 0.14 g., D-glucose 1.00 g., $NaHCO_3$ 2.20g., Phenol Red 0.004 g., H_2O (redistilled) to make 500 cc. Sterilized by filtration. This should be kept at 4° until use.

Solution-2 (S-2): $CaCl_2$ 0.20 g., $MgSO_4 \cdot 7H_2O$ 0.20 g. and H_2O (redistilled) to make 250 cc. Sterilized in an autoclave. This should be stored at room temperature.

Medium-I (M-I) and Medium-II (M-II): These should be prepared immediately before use. M-I consists of horse serum 96%, P-soln. 2%, and S-soln. 2%. M-II consists of equal parts of S-1 and S-2. For routine procedure, 85 cc. of M-I and 50 cc. of M-II are necessary to treat 5 samples at a time.

Cell Suspension: Two to 3 drops of 4-day-old ascites of the Yoshida sarcoma was added into a test tube containing 5 cc. of M-I. Cell population was counted under a microscope and a calculated volume of the cell suspension containing just 8×10^6 cells was transferred with a pipette into another flask and finally made up to 80 cc. with M-I. It therefore contained 1×10^5 cells/cc.

Serial Dilution of Compound: Immediately before beginning of experiment, a test compound was serially diluted with saline solution and 10-step dilution was enough for a routine procedure. A dilution ratio was usually chosen as shown in Table I.



Earle's solution

NaCl	KCl	$CaCl_2$	$MgSO_4 \cdot 7Aq.$	$NaH_2PO_4 \cdot Aq.$	d-Glucose	$NaHCO_3$	Phenol Red	Water to make (cc.)
g								
6.80	0.40	0.20	0.20	0.14	1.00	2.20	0.04	1000
4.25								500
2.55	0.40			0.14	1.00	2.20	0.04	250
		0.20	0.20					250

Chart 1.

Culture: First, 0.75 cc. of the test solution at certain concentration was mixed with 1.5 cc. of the cell suspension (1×10^5 cells/cc.) and then 0.75 cc. of M-II was added into this mixture. It was well mixed and divided equally into 3 test tubes (15 × 90 mm.) which were placed still in a vertical position at 37° in an incubator. This procedure was repeated with every test solution of different concentrations. In the final medium thus completed, cell population was one-half of the original suspension and concentration of the test solution was one-fourth the original dilution.

Microscopic Observation: After every 24, 48, and 72 hr. of incubation, each one of 3 tubes of the same concentration was taken out from the incubator and centrifuged. The cells were collected on an object glass and smeared, fixed with MeOH, and stained with Giemsa solution by the usual technique. Cytomorphological observation was carried out according to the technique and standard recommended by Yoshida[2] in their study on screening test using the Yoshida sarcoma rat and the minimum concentration at which the slightest but characteristic changes in figures after 48 hr. of incubation, corresponding to those at minimum effective dose (MED) in the rat experiment, is given in this paper as the minimum effective concentration (MEC).

Result

1) **N-Methyl-bis(2-chloroethyl)amine Hydrochloride** (HN_2)—The result is shown in Table I. MEC of this compound was determined as 2.5×10^{-4} m.*M* (0.0475 γ/cc.) in the final composition of the medium, which was reproducible in most of the experiments.

2) T. Yoshida, *et al.*: Gann, **45**, 484(1954).

Table I.

Compound	No. / γ/cc. / incubation	1	2	3	4	5	6	7	8	9	10	Control
		19	9.5	4.75	1.9	0.95	0.475	0.19	0.095	0.0475	0.019	0
CH_3-N(CH_2·CH_2·Cl, CH_2·CH_2·Cl·HCl) (HN_2) Mol. wt. 189.5	24 hr.	Cytolysis						Normal mitoses				
								55*		44	37	100
	48 hr.	Cytolysis						Abnormal mitoses →			Normal mitoses	
								205		283	228	100
	72 hr.	Cytolysis					→	Degeneration			Normal mitoses	
								42		70	63	100

MEC : 2.5×10^{-4} m.M (0.0475 γ/cc.)
* Mitotic index : Rate of mitosis (in %) to the control of each incubation period.

The mitotic indices at all stages of the culture are also shown in Table I. It was especially noteworthy that they were multiplied about twice of the contorl after 48 hr.'s incubation and, during the following 24 hr., it decreased again to about one-half of the control. However, the precise percentages did not appear constant in repeated trials, and furthermore it was not evident in case of other derivatives.

2) **Maximum Tolerance Concentration of Ethanol in the Medium**—Addition of EtOH to the medium in concentrations of 2.5% to 10% induced instant cytolysis and degeneration of tumor cells. At 1%, normal figure accompanied by unaffected mitosis was observed, although very slight cytoplasmic degeneration could be seen in a small portion of the cell population. No effect of EtOH was observed at a concentration of 0.5% or less.

3) **Effect of Ethanol on MEC of HN_2**—HN_2 was diluted with PS containing 4% of EtOH and serially diluted with PS without EtOH. It was mixed with cell suspension as described above to make a final concentration of EtOH in the complete medium 1% or less. MEC of HN_2 was also determined as 2.5×10^{-4} m.M, which was quite equal to that obtained without addition of EtOH. From these results, it is recommended to add EtOH to the medium in a permissible concentration, if a test compound has enough solubility for serial dilution.

4) **Picrate of HN_2**—The picrate (7.7 mg.) was dissolved in hot absolute EtOH (1 cc.) and diluted with PS exactly to 5 cc. One-half cc. of this mixture was then mixed with 4.5 cc. of PS and the mixture was serially diluted again with PS to make a series of test solutions. MEC here obtained was 2.5×10^{-4} m.M, which was equal to that of hydrochloride of HN_2. It may be worthy to note that a picrate of the amine gave the same value of MEC in this procedure as its hydrochloride, because, in animal experiment, efficacy of the two salts generally appeared different due to their different solubility in body fluid.

5) **6-Mercaptopurine**—This compound did not exhibit the typical effect in the routine procedure. To prolong the observation period, initial cell population in the complete medium was reduced to 2.5×10^4 cells/cc. and observation continued for 120 hr. Results are schematically demonstrated in Table II. Appearance of some huge cells was observed at comparatively high concentration but no

Table II.

Tube No.	Concn. m.M	Concn. γ/cc.	Incubation time (hrs.) 24	48	72	96	120
1	1×10^{-1}	15	No observable change	Cytolysis	Cytolysis	Cytolysis	Cytolysis
2	5×10^{-2}	7.5	〃	Huge cell	〃	〃	〃
3	2.5×10^{-2}	3.75	〃	〃	Huge cell	〃	〃
4	1×10^{-2}	1.5	〃	No observable change	〃	Huge cell	Huge cell
5	5×10^{-3}	0.75	〃	〃	〃	〃	〃
6	2.5×10^{-3}	0.375	〃	〃	〃	〃	〃
7	1×10^{-3}	0.15	〃	〃	No observable change	No observable change	No observable change
8	5×10^{-4}	0.075	〃	〃	〃	〃	〃
9	2.5×10^{-4}	0.0375	〃	〃	〃	〃	〃
10	1×10^{-4}	0.015	〃	〃	〃	〃	〃
Control	0	0	〃	〃	〃	〃	〃

characteristic morphological change in nuclei could be found. At higher concentration, only cytolysis appeared.

6) **Mitomycin C**—This compound was tested in a concentration range of 1,000~0.001 γ/cc. The effect was very similar to that of HN_2. Disturbance of mitotic process was the most pronounced feature in this case. Results are shown in Table III. MEC's of several other compounds are demonstrated in Table IV.

Table III.

Tube No.	Concn. γ/cc.	Incubation time (hrs.) 24	48	72
1	5	Cytolysis	Cytolysis	Cytolysis
2	2.5	〃	〃	〃
3	1	〃	〃	〃
4	0.5	Specific cytological effect	〃	〃
5	0.25	No observable change	〃	〃
6	0.1	〃	Specific cytological effect	〃
7	0.05	〃	〃	Specific cytological effect
8	0.025	〃	〃	〃
9	0.01	〃	No observable change	〃
10	0.005	〃	〃	No observable change
Control	0	〃	〃	〃

Table IV.

Compd. No.	Compound	Cytological effect	MEC m.M	MEC γ/cc.
24	HN_2-HCl	+	2.5×10^{-4}	0.05
556	HN_2-Picrate	+	2.5×10^{-4}	0.096
191	TEM	+	5×10^{-5}	0.01
305	Thio-TEPA	+	5×10^{-4}	0.095
263	6-Mercaptopurine	−		
242	8-Azaguanine	−		
239	Glucosamine	−		
555	5-Fluorouracil	−		
479	Carcinophillin	+		2.5 (U/cc.)
584	Mitomycin C	+		0.01

7) **Relationship between Growth Rate and Morphological Change by HN_2**—Seven culture tubes, each of which contained the Yoshida sarcoma cells (2.5×10^4 cells/cc.), were prepared. One of these was left as control and to the other 6, HN_2 was added at different concentrations. They were incubated together at 37° and after 24, 48, and 72 hr.'s incubation, number of cells in each tube was counted. The results are demonstrated in Fig. 1.

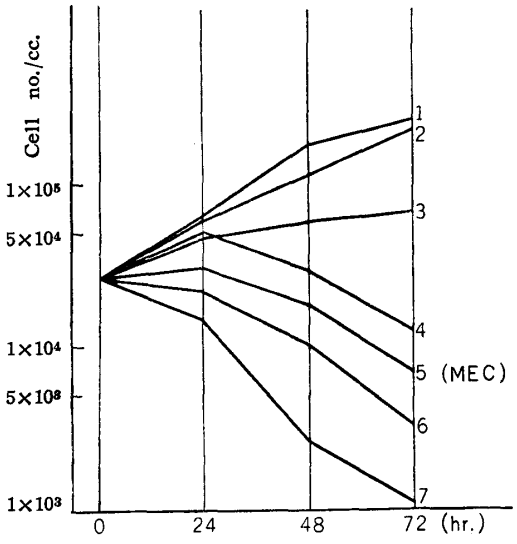


Fig. 1.

Compd.	HN_2 (m.M)
1	0 (for control)
2	1×10^{-5}
3	5×10^{-5}
4	1×10^{-4}
5	2.5×10^{-4} (MEC)
6	1×10^{-3}
7	1×10^{-2}

As seen in the figure, decrease in cell number corresponded to concentrations of HN_2 and curve 5 shows a limit concentration of morphological determination.

Discussion

The method is believed to be suitable for *in vitro* screening of the antitumor substances, the technique of cell culture is very simple and the state of growth is constant through repeated experiments. Contamination of the medium with bacteria can be practically avoided by addition of penicillin and streptomycin without any complicated aseptic technique. Optimal number of cells per cc. at inoculation should be not far more nor less than 5×10^4, because the less the cell population is, the better is the condition of cell growth, while too small a cell number is not sufficient to make a good smear preparation. The procedure of making a serial dilution is particularly arranged so as to keep a solution of a test compound always on the acid side until it comes in direct contact with the tumor cells. This caution is particularly necessary when the test compound is labile in a neutral or alkaline solution just like the derivatives of nitrogen mustard, which decomposes and transforms easily at pH-range from neutral to alkaline. Application of the method is further extended against the less soluble substances by confirming a limit of allowance of alcohol content in the medium.

Finally, cytomorphological determination of the effect of a compound is regarded to be very important in tissue culture screening, because it can draw a line between the real antitumor effect and non-specific toxicity of the antimitotic or radiomitotic substances. The other type of agent such as antimetabolites seems not always to exhibit any characteristic morphological aberration of the cells even at a high concentration. In order to test such a kind of compound, some cytochemical determination of the effect may be useful, for which efforts are now being continued.

Summary

A new and simplified method for screening of antitumor substances with *in vitro*-cultured tumor cells is presented. The effect of the test compound is determined by observation of the delayed appearance and long-lasting cytomorphological change of the cells through 72 hours of incubation. The method is especially suitable for screening of antimitotic or radiomitotic substances, because the real characteristic damage on nuclei can be discriminated from an acute unspecific cytotoxicity. By this technique, it is possible to avoid the error of taking a depression of growth rate caused by a mere cytolysis or by direct destruction as an index of the true anti-tumor effectiveness.

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