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73. Ayako Moriwaki : Studies on Carcinostatic Substances. XL.*1 Application of Cell Counting Method to the Screening of Antitumor Substances using the *in vitro*-cultured Yoshida Sarcoma Cells.

(*Iatrochemical Institute of Pharmacological Research Foundation**2)

Since 1956, the work of Eagle *et al.*,[1] Smith *et al.*,[2] and others on screening of antitumor compounds by inhibition grade of tumor cell growth *in vitro* have been repeatedly published.

The present author has also described in the preceding papers[3,4] the technique of *in vitro* culture of Yoshida sarcoma cell suspension and the screening of antitumor substances by means of this biological system. In those experiments, evaluation of the effect was done by the mere morphological observation by which the minimum effective concentration (MEC) of the compound was determined.

This paper deals with another method of *in vitro* screening, in which evaluation of the effect was carried out both by determination of the inhibition rate of cell proliferation and by morphological observation.

The composition of the medium was slightly changed from that the preceding work by lowering the content of horse serum from 50% to 20% and newly adding a certain quantity of lactalbumin hydrolysate, as will be described in the part of materials and method, so as to decrease the effect of quality of the serum on the growth rate, because of difficulty of keeping the serum in constant quality.

Indices of antitumor effect of compounds were represented by their concentration at which the cell growth was inhibited to 50% of the control and this is designated as the 50% inhibition concentration (IC_{50}). This method requires only 2 days for an individual specimen and needs no renewal of the medium of cultivation throughout the experiment.

Some antitumor compounds inhibit cell growth but does not induce any remarkable change in the figure of a cell, and this is why this new technique is preferred.

Materials and Methods

Horse serum (HS), Hank's balanced saline solution (HBS), physiological saline solution (PS), potassium penicillin-G-solution (p-soln.), and dihydrostreptomycin sulfate solution (s-soln) were prepared as described in the preceding paper.[3]

Solution H1 (SH1)——NaCl 7.5 g., KCl 0.8 g., $Na_2HPO_4 \cdot 12H_2O$ 0.24 g., KH_2PO_4 0.12 g., D-glucose 2.0 g., $NaHCO_3$ 0.5 g., Phenol Red 0.04 g., and H_2O (redistilled) to make 500 cc. Sterilized by filtration. This is kept at 4° until use.

Solution 2 (S2)——$CaCl_2 \cdot 2H_2O$ 0.26 g., $MgSO_4 \cdot 7H_2O$ 0.2 g., and H_2O (redistilled) to make 250 cc. Sterilized in an autoclave. This is stored at room temperature.

Hank's Balanced Saline Solution Containing Lactalbumin Hydrolysate (HL)——NaCl 2.8 g., $CaCl_2 \cdot 2H_2O$ 0.13 g., $MgSO_4 \cdot 7H_2O$ 0.1 g., $Na_2HPO_4 \cdot 12H_2O$ 0.06 g., KH_2PO_4 0.03 g., D-glucose 0.5 g., $NaHCO_3$ 0.125 g., Lactalbumin Hydrolysate (National Biochemicals Corp., U. S. A.) 7.15 g., and H_2O (redistilled) to make 500 cc.

Medium HI (MHI) and Medium HII (MHII)——These should be prepared immediately before use.

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*1 This paper constitutes a part of series entitled "Studies on Carcinostatic Substances" by M. Ishidate and Y. Sakurai. Part XXXIX : This Bulletin, **10**, 390 (1962).

*2 Designation now changed to Cancer Chemotherapy Section, Sasaki Institute. 26 Nishigahara, 1-chome, Kita-ku, Tokyo (森脇絢子).

1) H. Eagle, G. E. Foley : Am. J. Med., **21**, 739 (1956).
2) C. G. Smith, *et al.* : Cancer Research, **19**, 843 (1959).
3) M. Ishidate, *et al.* : This Bulletin, **7**, 690 (1959).
4) *Idem* : *Ibid.*, **7**, 873 (1959).

MHI consists of horse serum 40%, HL 56%, p-soln. 2%, and s-soln. 2%. MHII consists of equal parts of SHI and S 2. For routine procedure, 40 cc. of MHI and 21 cc. of MHII are necessary for one processing.

Cell Suspension——Quantity of medium to be added was adjusted to 1×10^5 cells/cc. in MHI.

Serial Dilution of Compound——Directly prior to the start of experiment, the test compound was serially diluted with saline at 0.5-logarithmic increments. Nine-steps dilution was usually enough for routine procedures but one more tube was added as a control to make ten tubes in all.

Culture——One-half cc. of the cell suspension (1×10^5 cells/cc.) was first mixed with 0.25 cc. of the serially diluted test solution and then with 0.25 cc. of MHII. The tubes were shaken gently, placed in a vertical position in an incubator, and stood still at 37°. Four sets of serial dilution were thus prepared and, after 48-hr. incubation, the cell number of each tube of three sets was counted and the fourth was used for morphological observation.

Cell Counting——Tubes were centrifuged and 2 cc. of a mixed solution of citric acid (0.1*M*) and Crystal Violet (0.05%) was added to the residue. The tubes were shaken at 37° for 30 min. in order to dissociate cell aggregates. After shaking, their aliquots were transferred to Bürker hemocytometer by pipette and cell number was counted under microscope.

Morphological Observation——The cells were collected on an object glass after centrifugation, smeared, fixed with MeOH, and stained with Giemsa solution. The minimum effective concentration (MEC) was determined as was described in the preceding paper.[4]

Result

Cell Number in Control Growth *in vitro* of Yoshida Sarcoma——The result of cell counting during incubation is shown in Fig. 1.

Mean value of cell number at each incubation period and standard deviations are shown in Table I.

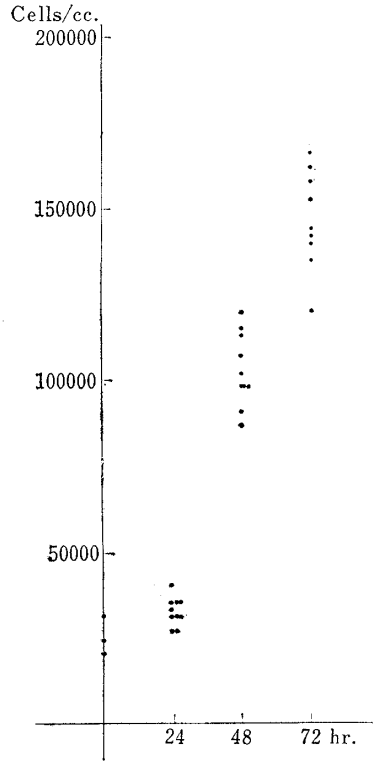


Fig. 1.

Table I.

	Mean number of cells	Standard deviation
Initial	25000 cells/cc.	
24 hr.	33000	± 4000
48 hr.	103000	± 11000
72 hr.	145000	± 15000

As seen in Table I, number of cells increased but standard deviation also increased as the incubation period was prolonged. Therefore it seemed better to stop incubation just after 48 hours, because, at this stage of incubation, cell number increased from four to eight folds of the initial, while standard deviation was yet tolerable. Moreover, certain compounds seem to manifest their effect for the first time after 48 hours of

incubation, so that the period of incubation adopted here should be preferable in this respect.

An Example of Assay with N-Methyl-bis(2-chloroethyl)amine Hydrochloride(HN_2)—— The ratio of cell number of the test tubes containing various concentrations of the compound of that of the control is demonstrated in Table II, and dose-response curve of this case is shown in Fig. 2.

Table II.

Compound	Concentration of HN_2 mM / Group		3.2×10^{-3}	1×10^{-3}	3.2×10^{-4}	1×10^{-4}	3.2×10^{-5}	1×10^{-5}	3.2×10^{-6}	1×10^{-6}	3.2×10^{-7}	Control
NH_2	48 hr. Growth rate of cells (%)	I	8.8	8.8	8.8	17.5	26.3	63.5	50.5	98.5	89.6	100.0
		II	6.6	2.2	8.8	17.5	28.4	70.0	76.5	72.1	89.6	100.0
		III	4.4	6.6	8.8	13.1	39.4	41.6	81.0	98.5	78.8	100.0
M.W. 189.5	48 hr. C.E.*	IV	+	+	+	±	∓	−	−	−	−	−

* C.E. Cytological effect

MEC of this compound was determined as 1×10^{-4} mM (0.019 γ/cc.)

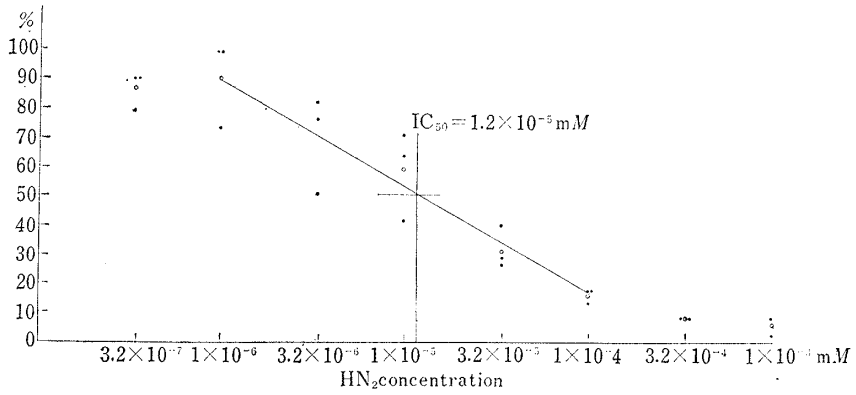


Fig. 2.

The dose-response curve of HN_2 was linear between the concentrations of 1×10^{-6} mM and 1×10^{-4} mM and the representative equation and IC_{50} were calculated by the statistical method. The analysis of Variance is summarized in Table III.

Table III. Analysis of Variance

Source of variation	Sum of squares	Degree of freedom	Variance	Variance ratio (F_0)
Regression	$S_R=10300.827$	1		
Deviations from regression	$S_{DR}=130.16$	3	43.339	$F_3^{10}=0.277$
Between doses	$S_{YY}=10430.99$	4		
Within deses	$S_e=1561.53$	10	156.153	
Total	$S_D=11992.52$	14		

$$Y=53.0-18.5X$$

Factorial Coefficient $=2\log(A/1\times10^{-5})$

A = concentration

$IC_{50}=1.2\times10^{-5}$ mM

Range : $(2.0\sim0.7)\times10^{-5}$ mM

The Reproducibility of Results with HN_2—— Results of assay of HN_2, repeated six times during 6 months, are summarized in Table IV.

Table IV.

Date	MEC (mM)	IC_{50} (mM)	Date	MEC (mM)	IC_{50} (mM)
October 1, 1959	1×10^{-4}	1.2×10^{-5}	October 10, 1959	1×10^{-4}	1.0×10^{-5}
October 5, 1959	1×10^{-4}	1.6×10^{-5}	October 13, 1959	1×10^{-4}	1.1×10^{-5}
October 6, 1959	1×10^{-4}	2.0×10^{-5}	April 9, 1960	1×10^{-4}	1.9×10^{-5}

Maximum Tolerance Concentration of Ethanol in the Medium——Water-insoluble compounds were dissolved in aqueoues organic solvents. The inhibitory effect of ethanol on the growth of the *in vitro*-cultured Yoshida sarcoma was determined and its result is shown in Table V. From these data, the final concentration of ethanol should be less than 0.3% of the medium.

Table V.

Compound	Concentration of C_2H_5OH / Group		1 3%	2 1%	3 0.3%	Control 0
EtOH	48 hr. Growth rate of cells (%)	I	0	50.0	100.0	100.0
		II	0	40.0	115.5	100.0
		III	0	46.0	105.1	100.0

Inhibitory Activity of Other Compounds——The activity of compounds assayed by this technique is demonstrated in Table VI (In all cases, the linearity between a certain range of concentrations was proved).

The graphical demonstration of several case of them are given in Figs. 3 and 4.

Discussion

The data presented above show that the assay of IC_{50} with the *in vitro*-cultured Yoshida sarcoma is more sensitive than the morphological determination of cell damages (MEC) and the results are also more reproducible. But, of course, the morphological determination gives further informations as to the quality of their antitumor activity than the determination of mere cell counting and, for this reason, a concomitant determination of MEC with IC_{50} is recommended.

However, so far as is known with alkylating agents, there has been found a good parallel correlation between MEC and IC_{50} in one compound.

Eagle and Smith[2] used the tumor strains successively cultured *in vitro* and determined cell number by a quantitative determination of protein content of the whole cell population. Above two methods require 7 and 3 days, respectively, for assay of one specimen, whereas the present procedure needs only 2 days without renewal of the medium throughout the experiment.

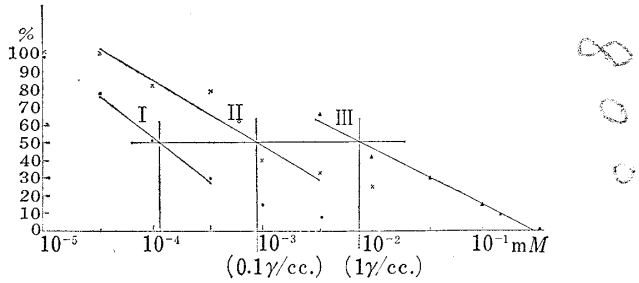
This method is believed to have merits in convenience of practice and also in giving an index of effect corresponding to that obtained with *in vivo* tumor of the same kind.

The way of cell counting under microscope is often preferable, because it can afford more exact value even with a small number of cell population, with which protein determination may not be successful. Moreover, inclination of the straight line (tangent) in the dose-response curve seems to be characteristic to the individual compound as described by Treffers[5] in his work on dose-response curves in microbial-antibiotic assays and accordingly there may be a possibility of gaining some informations about the mode of action of compounds by comparison of tangent values of their straight dose-response curve. In fact, it was found that the inclination of HN_2 was very different from that of colchicine.

5) H. P. Treffers : J. Bacteriol., **72**, 108 (1956).

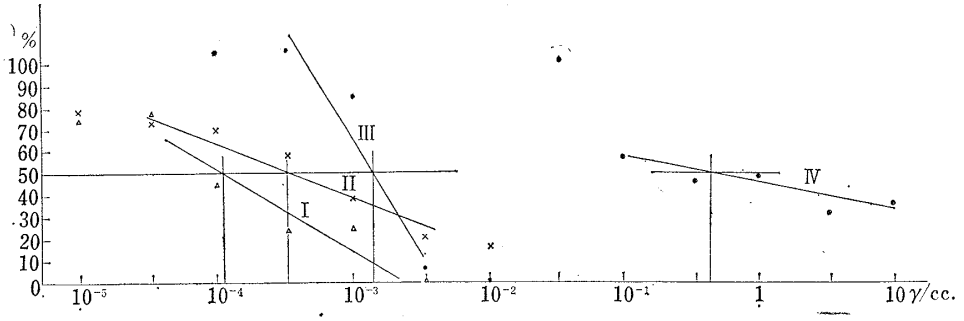
Table VI.

Compound	MEC	IC_{50}	Range	Factorial coefficient	Linear range	Equation
CH_3-N$\langle^{CH_2CH_2Cl}_{CH_2CH_2Cl}$ (HCl) (HN_2)	1×10^{-4} mM	1.2×10^{-5} mM	$2.0\sim0.7\times10^{-5}$ mM	$2\log(A/1\times10^{-5})$	$1\times10^{-4}\sim1\times10^{-6}$ mM	$Y=53.0-(18.5\pm6.0)x$
H_2NOC-CH_2-N$\langle^{CH_2CH_2Cl}_{CH_2CH_2Cl}$ (HCl)	3×10^{-3} mM	1.2×10^{-4} mM	$1.4\sim1.1\times10^{-4}$ mM	$2\log(A/1\times10^{-4})$	$3.2\times10^{-4}\sim3.2\times10^{-5}$ mM	$Y=53.0-(25.4\pm3.9)x$
NC-CH_2CH_2N$\langle^{CH_2CH_2Cl}_{CH_2CH_2Cl}$ (HCl)	3×10^{-3} mM	1.2×10^{-4} mM	$2.0\sim0.7\times10^{-4}$ mM	$2\log(A/1\times10^{-4})$	$1\times10^{-3}\sim1\times10^{-5}$ mM	$Y=52.3-(17.1\pm5.7)x$
CH_3-N$\langle^{CH_2CH_2Cl}_{CH_2CH_2Cl}$ (O)	(−)	4.9×10^{-4} mM	$12\sim2.0\times10^{-4}$ mM	$2\log(A/1\times10^{-3})$	$3.2\times10^{-3}\sim3.2\times10^{-4}$ mM	$Y=37.4-(20.5\pm17.2)x$
Mitomycin C	$1\times10^{-2}\,\gamma$/cc.	$3.1\times10^{-4}\,\gamma$/cc.	$9.3\sim2.1\times10^{-4}\,\gamma$/cc.	$2\log(A/3.2\times10^{-4})$	$3.2\times10^{-3}\sim3.2\times10^{-5}\,\gamma$/cc.	$Y=50.8-(12.1\pm4.5)x$
Actinomycin C	(−)	$1.1\times10^{-4}\,\gamma$/cc.	$2.7\sim0.4\times10^{-4}\,\gamma$/cc.	$2\log(A/3.2\times10^{-4})$	$3.2\times10^{-3}\sim3.2\times10^{-5}\,\gamma$/cc.	$Y=33.8-(17.5\pm8.3)x$
Sarcomycin	(−)	$11\,\gamma$/cc.	$15\sim8\,\gamma$/cc.	$2\log(A/10)$	$100\sim1\,\gamma$/cc.	$Y=52.6-(29.9\pm6.0)x$
6-Mercaptopurine	(−)	8.3×10^{-3} mM	$14.6\sim4.7\times10^{-3}$ mM	$2\log(A/1\times10^{-2})$	$1\times10^{-1}\sim1\times10^{-3}$ mM	$Y=46.8-(19.5\pm6.8)x$
5-Fluorouracil	(−)	9.0×10^{-4} mM	$13.9\sim5.9\times10^{-4}$ mM	$2\log(A/3.2\times10^{-4})$	$3.2\times10^{-3}\sim3.2\times10^{-5}$ mM	$Y=67.3-(17.8\pm3.8)x$
Aminopterin	(−)	$0.45\,\gamma$/cc.	$2.2\sim0.008\,\gamma$/cc.	$2\log(A)$	$10\sim0.1\,\gamma$/cc.	$Y=45.5-(6.4\pm5.8)x$
Colchicine	$3\times10^{-3}\,\gamma$/cc.	$1.5\times10^{-3}\,\gamma$/cc.	$2.5\sim0.9\times10^{-3}\,\gamma$/cc.	$2\log(A/1\times10^{-3})$	$3.2\times10^{-3}\sim3\times10^{-4}\,\gamma$/cc.	$Y=66.1-(50.1\pm21.8)x$
4-Nitroquinoline N-oxide	(−)	1.6×10^{-6} mM	$2.1\sim1.3\times10^{-6}$ mM	$2\log(A/1\times10^{-6})$	$3.2\times10^{-5}\sim3.2\times10^{-6}$ mM	$Y=64.6-(34.2\pm9.4)x$
M. H.	(−)	$3.3\,\gamma$/cc.	$5.3\sim2.1\,\gamma$/cc.	$2\log(A/3.2)$	$10\sim1\,\gamma$/cc.	$Y=53.7-(41.7\pm22.5)x$
Azo 106	(−)	$35\,\gamma$/cc.	$52\sim24\,\gamma$/cc.	$2\log(A/32)$	$320\sim32\,\gamma$/cc.	$Y=49.3-(29.5\pm8.5)x$



I : H_2N-OC-CH_2N$\langle^{CH_2CH_2Cl}_{CH_2CH_2Cl}$·HCl ● Mean value of I
II : 5-Fluorouracil × Mean value of II
III : 6-Mercaptopurine ▲ Mean value of III

Fig. 3.



I : Actinomycin C △ Mean value of I
II : Mitomycin C × Mean value of II
III : Colchicin ● Mean value of III
IV : Aminopterin ⊙ Mean value of IV

Fig. 4.

It is believed therefore that this technique is not only useful in screening unknown antitumor compounds but also in analyzing condition of manifestation of action or mode of action of certain compounds. Determination of resistant grade of resistant strains of Yoshida sarcoma was also defined by comparing IC_{50} of the resistant and the normal strains by this technique.

The author is very grateful to Prof. Emeritus M. Ishidate, Prof. T. Yoshida and Dr. Y. Sakurai for their kind guidances throughout the course of this investigation and also to Drs. H. Satoh, H. Imamura, and Mrs. H. Imai for their technical collaboration. She is very thankful to Dr. S. Matsushima for his kind guidance of the statistical analysis.

Summary

A new method of screening of antitumor compounds employing Yoshida sarcoma cell suspension cultured *in vitro* was presented. Evaluation of the antitumor effect was carried out by determination of cytomorphological change and inhibition of cell proliferation by cell counting.

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74. Toyozo Uno and Koichiro Miyajima : Determination of Surface-active Agent. III.[*2] Volumetric Determination of Anionic Surface-active Agent using Neutral Red as an Indicator.[*3].

(*Faculty of Pharmacy, Kyoto University*[*1])

Titrimetric determination of surface-active agents using dyes has been studied and widely used, because of simplicity of procedure, accuracy of results, and convenience in practical use. Two series of volumetric methods of surfactant determination has been known, namely, as Hartley's and Epton's methods.[1,2]

Hartley[1] reported the determination of cationic surface-active agent (CSAA) with anionic surface-active agent (ASAA) using Bromophenol blue as an indicator in ammonia alkaline medium. This method depends upon the difference of the color of Bromophenol blue-CSAA complex (blue) and alkaline Bromophenol blue (blue purple), however the recognition of the end-point is not easy, because of the similarity of these two colors.

Epton[2] also determined the ASAA with CSAA using Methyleneblue and organic solvents, such as chloroform or ethylene dichloride. Methyleneblue forms a complex with ASAA, which is insoluble in water and soluble in organic solvents. This method has been studied by many workers.[3~6] Recently Aoki and Iwayama[7] reported the determination of ionic surface-active agents using Bromocresol green-Neutral red (NR) com-

*1 Yoshida-konoe-cho, Sakyo-ku, Kyoto (宇野豊三, 宮島孝一郎).
*2 Part II : This Bulletin, **9**, 326 (1961).
*3 This work was presented at the Kinki branch meeting of Pharmaceutical Society of Japan, February, 1961.

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