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123. Morizo Ishidate, Yoshio Sakurai, Hiroshi Imamura, and Ayako Moriwaki: Studies on Carcinostatic Substances. XX.*1 Studies on the Culture of Yoshida Sarcoma Cells *in vitro*.

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In the course of studies on carcinostatic substances using experimental tumor animals, it is of course important to investigate their life prolongation effect on the tumor-bearing animals. It seemed of more importance to examine closely cytological changes in appearance caused by the test compounds and discuss about correspondency of the change of appearance to the effect of life-prolongation. The characteristic change of the image of cells, especially the cells in mitotic state, can be employed without question as an index of cell damage induced by the chemical compound.

To this end, it is quite necessary that such a morphological observation of the cell effect is undertaken under definite experimental conditions, which can be chosen at will according to the purpose of the experiment. The conclusion is that it is more preferable to use the *in vitro*-cultured tumor cells for this purpose than to work with the ascites tumor *in situ*, because, in the former case, either the time of contact, method of application, dosage and concentration or the number of cells, period of incubation, and constituent of the medium can be fixed so as to meet a definite purpose.

As to the tissue culture of Yoshida sarcoma, the works of Lettré,[1] Schleich,[1,2] Tobioka,[3] and others have been published. Lettré and Schleich had stated that the Yoshida sarcoma could proliferate only when the tumor cells were kept in contact with the fibroblasts of rat in the medium, but in order to apply tissue culture method in cytomorphology or biochemistry, it was of course preferable to avoid any accompaniment of cells other than the tumor in one culture. An object of the present study was therefore to establish a method of keeping a constant growth of the tumor cells alone *in vitro* for a period which was long enough for some cytomorphological and biochemical research to be made of the tumor. In a previous work,[4] it was shown that continuous growth of the cells *in vitro* seemed to be limited by an area of the bottom surface of flask on which they lay. From this aspect, the initial number of cells in one tube was adjusted to 1×10^4 to 5×10^4 in the present experiment.

The medium was also modified as described below and a constant growth of the Yoshida sarcoma alone *in vitro* was successfully maintained without refeed for 72~120 hours. It should be noted that removal of embryonal extract from the composition of medium resulted in a better state of culture in case of Yoshida sarcoma. The technique of culture, rate of growth, and some biological behaviors of the tumor thus cultured are presented in this paper.

Materials and Methods

1) **Horse Serum**—A sterile horse serum was inactivated by heating at 56° for 30 mins. and stored at −10° until use.

2) **Bovine Serum**—A sterile bovine serum was also inactivated similarly as above.

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*1 Part XIX: This Bulletin, **6**, 169(1958).

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1) H. Lettré, A. Schleich: Naturwiss., **41**, 505(1954).
2) A. Schleich: *Ibid.*, **42**, 50(1955).
3) M. Tobioka, *et al.*: Gann, **41**, 37(1950).
4) Y. Sakurai, *et al.*: *Ibid.*, **48**, 534(1957).

3) **Chick Embryo Extract**—With precautions to avoid bacterial contamination, a certain number of 9-day-old embryos was collected and minced to tissue pulp in a syringe. The pulp was mixed with an equal part of Earle's balanced saline solution and the mixture was kept overnight in a refrigerator at 4°. It was then centrifuged at 3,000 r.p.m. for 30 mins. and the separated supernatant fluid was stored at −10° until use.

4) **Earle's Balanced Saline Solution**—The composition of Earle's balanced saline solution was as follows :

	(g.)		(g.)
NaCl	6.80	D-Glucose	1.00
KCl	0.40	$NaHCO_3$	2.20
$CaCl_2$	0.20	Phenol Red	0.05
$MgSO_4 \cdot 7H_2O$	0.20	Water (redistilled in all-glass app.)	
$NaH_2PO_4 \cdot H_2O$	0.14	to make 1 L.	

The solution was sterilized by filtration and stored at 4° until use.

5) **Hanks' Balanced Saline Solution**—The composition of Hanks' balanced saline solution was as follows :

	(g.)		(g.)
NaCl	8.00	KH_2PO_4	0.06
KCl	0.40	D-Glucose	1.00
$CaCl_2$	0.20	$NaHCO_3$	0.25
$MgSO_4 \cdot 7H_2O$	0.20	Phenol Red	0.02
$Na_2HPO_4 \cdot 2H_2O$	0.06	Water (redistilled in all-glass app.) to make 1 L.	

The solution was sterilized by filtration and stored at 4° until use.

6) **Physiological Saline Solution**—0.85% NaCl.

7) **Potassium Penicillin G Solution**—10,000 U/cc.

8) **Dihydrostreptomycin Sulfate Solution**—10 mg./cc.

9) **Lactalbumin Enzymatic Hydrolysate**—The stock solution in Earl's balanced saline solution (5 g./L.) was sterilized by filtration and kept below 4° until use.

10) **The Yoshida Sarcoma Cell Suspension**—The tumor cells for inoculation were taken directly from the ascites of tumor-bearing albino rat. Two to three drops of the ascitic fluid from 4-day-old-tumor rat were added into a test tube containing a certain volume of the culture medium. Cell population in the test tube was adjusted by counting of the cells to about 5×10^4 cells/cc. by dilution with the same medium.

11) **Culture Vessel**—Small test tubes (15 × 90 mm.), which could be sealed with double rubber stoppers during cultivation, were used.

12) **Culture Method**—The culture tubes, each containing 1 cc. of the cell suspension in the medium, were kept standing still in a vertical position at 37° in an incubator.

13) **Smear Preparation**—At the end of incubation, the test tube was centrifuged at 2,000 r.p.m. for about 20 sec. The supernatant fluid was removed by decantation, and holding the tube up-side down in one hand, the precipitated cells were collected by a capillary pipette. The content of the pipette was blown out and smeared on the object glass, which was successively fixed with methanol and stained with Giemsa solution after the usual procedure.

Results and Discussion

I) Medium for Culture

In order to determine the optimal composition of the medium, 19 kinds of media having different composition were tested, which are shown in Table I. The vitality of the cultivated cells in each medium was checked morphologically by observing the rate of division, and the grade and rate of degeneration.

The results may be summarized as follows :

1) There was no difference in the growth-promoting effect between horse serum and bovine serum.

2) No better result was obtained on adding chick embryo extract than in case of the medium containing only the serum and saline.

3) It was found that a mixture of nearly the same portions of the serum and the balanced saline solution was the most preferable.

4) The fresh serum of either horse or bovine, without being inactivated, seemed to

Table I. Composition of the Medium Tested* (%)

No.	CEE	HS	EBS	No.	CEE	HS	BS	EBS	HBS	LAH (final)
										w/v%
1	20	40	40	11	20		40	40		
2	10	40	50	12	10		40	50		
3	5	45	50	13	5		45	50		
4	2	48	50	14	2		48	50		
5		50	50	15			50	50		
6		40	60	16		50			50	
7		30	70	17		50[a]		50		
8		20	80	18		20			80	0.4
9		10	90	19		5			95	0.5
10			100							

a) Fresh horse serum without inactivation

* Abbreviations used

CEE : Chick embryo extract
HS : Horse serum
BS : Bovine serum
EBS : Earle's balanced saline solution
HBS : Hanks' balanced saline solution
LAH : Lactalbumin hydrolysate

have a certain unfavorable effect on proliferation of the cells.

5) Amount of the serum could be reduced from 50% to 20% without giving an unfavourable influence upon the cell growth, if lactalbumin hydrolysate is added to 0.4% final concentration of the medium (*No. 18). In the case of medium No. 19, in which the serum was further reduced to only 5%, the growth of the cell was markedly retarded and a large number of the degenerated figures was observed in spite of increasing lactalbumin hydrolysate up to 0.5%.

6) Hanks' solution was equally useful as Earle's solution in all the experiments.

7) Addition of penicillin or streptomycin, the concentration of which has already been described, was proved to have no practical effect on the growth rate or appearance of the tumor cells.

From these results, the medium indicated in Table II was adopted for the culture of Yoshida sarcoma.

Table II. Final Concentration of Medium

Horse serum	48%
Earle's balanced saline solution	50%
Physiological saline solution	2%
containing	
Penicillin G (K salt)	100 U/cc.
Dihydrostreptomycin sulfate	100 γ/cc.

It was proved that the morphological view of the Yoshida sarcoma cells never became deteriorated in this medium through the course of 96 hours' incubation and they looked as vital as those proliferated in the peritoneal cavity of a rat.

II) Mitotic Percentage

As an index of the vitality of the cell, mitotic percentage of the *in vitro*-cultured cells was checked by counting with the Giemsa-stained preparation. For this purpose, a number of mitosis among 1,000 cells, which were chosen at random on each of the smeared and stained object glasses, was counted under a microscope at 600 magnification. The percentages are shown in Table III.

During incubation, there was no tendency for mitotic percentage to decrease with passage of time until 72 hours and the average value of all experiments was found to be approximately the same as that of Yoshida sarcoma *in situ*, the number of which was

Table III. Mitotic Percentage of *in vitro*-cultured Yoshida Sarcoma Cells

Incubation time (hr.) \ Expt. No.	1	2	3	4	5	6	average
24	4.4	1.5	2.8	2.2	2.3	2.1	2.6
48	1.8	2.7	2.7	1.9	2.0	1.4	2.0
72	2.7	1.9	3.7	2.2	1.8	1.3	2.3
average	3.0	2.0	3.1	2.1	2.0	1.6	2.3

once reported by Yoshida[5] as 2.25% in average.

III) Growth Rate

The following experiment was carried out to ascertain the rate of actual increase of cell number during incubation. The total cell number in each of the culture tubes after a certain period of incubation was counted by Bürker's hemacytometer under a phase-contrast microscope at 100 magnification without fixing.

Table IV. Cell Proliferation after Incubation

Expt. No.	Initial no. of cells in 1 cc. of medium	No. of cells in 1 cc. medium after incubation		
		24 hr.	48 hr.	72 hr.
1	2.5×10^4	6.3×10^4	18.2×10^4	25.5×10^4
2	10.6×10^4	27.4×10^4	36.9×10^4	29.7×10^4

As shown in Table IV, the tumor cells actually increased in number during the period of incubation of 24~72 hours, but its growth rate during 72 hours depended on the initial concentration of the cells in this medium. It seemed that the increase of the cell number ceased at the similar level of the concentration of about 30×10^4/cc. in both experiments, at whatever population they began to grow. The culture fluid in the final stage of growth was then centrifuged at 2,000 r.p.m. for 20 seconds and the supernatant was removed. The cellular residue was suspended in a calculated amount of the fresh medium so as to make its cell population 5×10^4/cc. and again incubated. The passage of the culture could be thus continued if the medium was refed every 2 or 3 days, but growth rate seemed to retard gradually through repetition of passage. It was perceived that the cells cultured by this means showed no morphological degeneration even after 7 days' incubation.

IV) Back-transplantation of the Tumor cultured *in vitro* onto Rat

Each 0.5 cc. of the 5-, 10-, and 14-day culture fluid of this tumor was inoculated into peritoneal cavity of 2 rats. These rats died without exception by tumor growth, as demonstrated in Table V, and the progress and behavior of the tumor in the host observed by

Table V. Back-transplantation on Rat of *in vitro*-cultured Yoshida Sarcoma Cells

Incubation time *in vitro* (days)	5	10	14
Transplantability	2/2	2/2	2/2

autopsy or the microscopic figures of the cells taken from these animals agreed well with those of the typical Yoshida sarcoma rat. In conclusion, the cultured cells of the Yoshida sarcoma by this means can be estimated to have nearly the same vitality at least for three days after inoculation as those of the same tumor *in vivo*. It was therefore anticipated that not only screening and analysis of the anticancer effect of the substances but various biochemical examination of tumor cells may become easier and

5) T. Yoshida: "Yoshida Nikushu," (1949). Nara-Shobo, Tokyo.

more precise if this simple method of *in vitro*-culture is applied.

V) *In vitro*-Culture of Ascites Hepatomas

Nineteen kinds of rat ascites hepatoma*[3] were also investigated as to their behavior *in vitro*, using a similar medium and technique as those described above for Yoshida sarcoma. Seventy-two hours after inoculation, the microscopic observation of the cells was undertaken in every case. All hepatomas, shown in Table VI, seemed to have less tendency to adapt to this *in vitro*-condition than the Yoshida sarcoma. The tumor cells belonging to the group A of the Table maintained almost normal and sound appearance under microscope after 72 hours' incubation. The appearance of tumor cells belonging to the group B was always accompanied by an unnegligible grade of degeneration and those of the group C appeared completely degenerated at the end of 72 hours' incubation.

Table VI.

Group	Ascites hepatoma			
A	AH 601	AH 7974	AH 66	
B	AH 423	AH 66F	AH 62	AH 602
C	AH 130	AH 322	AH 63	AH 21
	AH 99	AH 318	AH 149	AH 39
	AH 414	AH 13	AH 49	AH 364

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Summary

A method of culturing the Yoshida sarcoma *in vitro* was established. The tumor cells maintained constant vitality and growth for at least 72 hours in a simple and convenient medium. The method is believed to be applicable in the wide field of research on cancer chemotherapy.

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*[3] They were induced on rats by feeding with the azo dye at Sasaki Institute for Medical Research, Tokyo.