Acta Medica Okayama

Volume 20, *Issue* 5 1966 *Article* 2

OCTOBER 1966

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Abstract

For the purpose to clarify whether minimal catalatic activity exists in Japanese acatalasemic cells or not and the manner how extrinsic hydrogen peroxide affects the acatalasemic cells, the author performed tissue cultures using the skin specimens from four acatalasemic persons affected with Takahara's disease and studied the nature of these cultured cells. The results are summarized as follows. 1. Between normal and acatalasemic cultured cells, no morphological differences could be seen and the growth rate of these cell-lines was similar to one another. 2. On the activity of succinoxidase and cytochrome oxidase there could be observed no difference between normal and acatalasemic cells. 3. In each acatalasemic cell line the minimal catalatic activity was observed and it seemed that this activity has an important role in decomposing hydrogen peroxide under normal metabolic pathway. 4. After treating with 10-4M hydrogen peroxide, respiratory enzyme activities and the growth rate in the acatalasemic cells were markedly disturbed, while in normal cells these remained almost intact. 5. There could be observed no differences between normal and acatalasemic cultured cells after X-ray irradiation (200 to 600 r) on the succinoxidase activity, catalatic activity and growth rate.

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*PMID: 4227145 [PubMed - indexed for MEDLINE]

Acta Med. Okayama 20, 193—202 (1966)

NATURE OF CULTURED CELLS OF THE SKIN FROM ACATALASEMIC INDIVIDUALS WITH TAKAHARA'S DISEASE

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Received for publication, September 16, 1966

Acatalasemia first described by TAKAHARA[1] in 1949, is a rare hereditary constitutional abnormality, characterized by deficiency of catalase in the blood.

To make clear the biological state of this acatalasemia, KITAMURA *et al.*[2] and KROOTH *et al*[3]. carried out successfully the tissue cultures with Japanese acatalasemic skin materials and they showed that the acatalasemic cells cultured *in vitro* inherited the nature of acatalasemia, the deficiency of catalase activity. KROOTH *et al.* reported that there were no differences between normal and acatalasemic cultured cells on the growth rate after exposure to various concentrations of hydrogen peroxide.

Besides these, AEBI *et al*[4]. also carried out the tissue culture with Caucasian acatalasemic skin material and proved the existence of residual catalase activity in these cells.

The purpose of this study is to clarify whether minimal catalatic activity exists in Japanese acatalasemic cells or not, and to study more precisely on the manner how extrinsic hydrogen peroxide affects the acatalasemic cells.

MATERIALS AND METHODS

The skin specimens (about 1 cm^2) obtained by biopsy from four acatalasemic individuals with oral gangrene, shown in Fig. 1 by arrow, and from three normal persons as the control served as materials.

1. Tissue culture : Tissue cultures were carried out by the method of HARNDEN[5]. The growth medium employed for the cultures was prepared by Harnden's prescription**. In the primary culture, when the fusiform fibroblasts began to proliferate around the original tissue from about seventh day, the

This study was supported by the Research Grant (AM 06793-02 GEN) from the National Institutes of Health, the U. S. Public Health Service.

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** Harnden's prescription: 60% of T. C.-Medium 199, 20% of human serum, and 20% of chick embryo (9th day) extract.

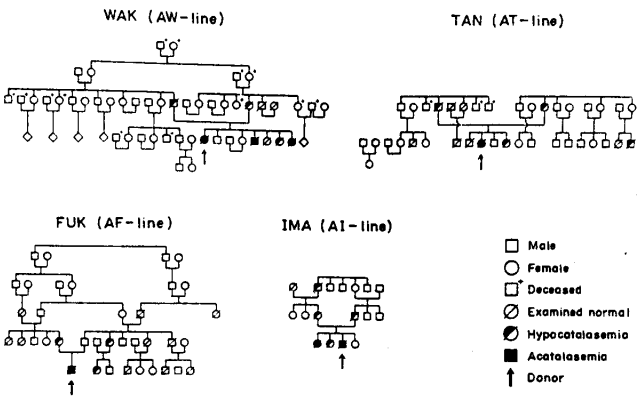


Fig. 1 Pedigrees of Four Acatalasemic Skin Donors

medium was changed once. After about two weeks of the primary culture the proliferated cells were removed from the wall of the test tubes by tripsinization with the use of 300 H. U. M. (Mochida Co.) in Hank's solution, transferred in small or large square bottles ($3\times3\times5$ cm, or $5\times5\times15$ cm) and subcultured.

2. Measurement of the growth rate : Three large bottles of the cells were subcultured equally in 18 small bottles. Then, three of them were removed for measurement of cell protein every other day up to the tenth day of subculture and the growth curve was drawn.

3. Measurement of cell protein : Lowry-Sutherland's method[6] was used in each experiment for the protein measurement.

4. Measurement of catalase activity : Acatalasemic cells from 5 large bottles and normal cells from one bottle were harvested by gum-rubber, and these respective cells were washed three times with 0.9% NaCl solution. The washed cells were suspended in 10 ml of 0.01 M phosphate buffer (pH 6.8) and homogenized by the glass homogenizer (Potter-Evelin type) with addition of one drop of Tween 20. The homogenate was centrifuged at 3,500 rpm for 5 minutes, and the supernatant thus obtained served as material for enzyme assay. *a) Manometric method :* Modified method of FUJITA and KODAMA[7] was employed. In this experiment, 3 ml of the above supernatant were put into the main compartment of Warburg's cup. *b) Perborate method :* Feinstein's method[8] was applied to catalase assay for the above supernatant.

5. Measurement of succinoxidase activity : The cells taken from 5 large bottles by tripsinization were washed three times with 0.9% NaCl solution and suspended in 75 ml of the growth medium. This suspension was pipetted into 15 small bottles in equal volume and incubated at 37°C for 3 days until the cells grew in a monolayer form. Then, removing the medium, the monolayer-cultured cells were washed three times with 0.9% NaCl solution and used for

enzyme assay, without detaching from the wall of the bottles. For one series of experiments, 15 small bottles above mentioned were used: 3 of them were used for the measurement of cell protein contents, the rest 12 bottles were divided into two groups, 6 in each; one group was used for the assay of the succinoxidase and the other for the measurement of endogenous dehydrogenase activity. The cells in each bottle were overlaid with 1.2 ml of the reagent mixture* and incubated at 37°C for one hour. Then the reaction was stopped by addition of 1 ml of 10% formalin solution. The diformazan produced in 3 bottles was extracted with 4 ml of aceton-ether (1 : 1), and the optical density of the extract was measured at 520 mμ by the spectrophotometer, Hitachi Perkin-Elmer 139 type.

6. Measurement of cytochrome oxidase activity: The cells harvested from 5 large bottles were suspended in 3 ml of 20 mM Tris-HCl buffer solution (pH 7.0) containing 10 mM KCl and homogenized with the glass homogenizer with addition of 1 drop of Tween 20. This homogenate was centrifuged at 3,500 rpm for 5 minutes and the supernatant thus obtained was used as the enzyme solution. The reagents** were added into the oxymeter successively, and the oxygen consumption at 37°C was measured.

7. Treatment with hydrogen peroxide on the cells: The effect of hydrogen peroxide on the growth rate and respiratory enzyme activities of the cultured cells were examined following the methods described in the items 2, 5 and 6, after treatment with 10^{-4}M hydrogen peroxide diluted with physiological saline solution at 37°C for 10 minutes. The treatment with hydrogen peroxide on the cells was as follows: in the case of the succinoxidase activity assay, the monolayer-cultured living cells in small bottles were at first overlaid with 1.5 ml of the above mentioned 10^{-4}M hydrogen peroxide, and in the measurement of cytochrome oxidase activity or the growth rate, the harvested and washed cells were previously suspended in the 10^{-4}M hydrogen peroxide solution.

8. Experiments of X-ray irradiation: To know the sensitivity of the cells to X-ray irradiation the growth rate, catalatic activity and succinoxidase activity were examined. In the succinoxidase activity assay, the

Table 1 Conditions of X-ray Irradiation

Apparatus	Toshiba KXC-18 Type
Filter	0.5 cm Cu and 0.5 cm Al
Distance	50 cm
Dose rate	71 r/min., 200 KVP, 25 mA

* The reagent was a mixed solution of 0.2% neotetrazolium solution, 0.2M sodium succinate solution (or 0.9% NaCl solution, for the assay of endogenous dehydrogenase activity) and 0.1M phosphate buffer (pH 7.6), each combined with an equal volume.

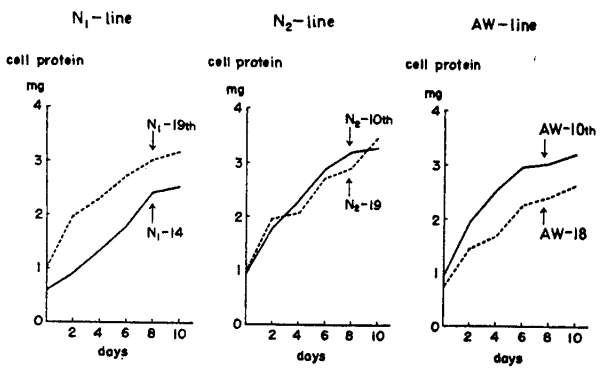
** The reagents were 1.8 ml of the enzyme solution, 0.002 ml of 1% Antimycin A in absolute ethanol solution, 0.3 ml of 0.1M *l*-ascorbate solution, 0.02 ml of 0.01M tetramethyl-paraphenylenediamine solution and 0.02 ml of cytochrome *c*.

On the prescription of these reaction medium the author is indebted to Prof. T. ODA, Cancer Institute of Okayama University Medical School.

monolayer intact cells were overlaid with 1 ml of air-saturated 0.9% NaCl solution and irradiated. For the measurements of catalatic activity and growth rate, the cells were suspended in air-saturated 0.9% NaCl solution and then irradiated. The conditions of irradiation are shown in Table 1. After the irradiation, the cells were washed three times with 0.9% NaCl solution in each case and served as materials for each experiment.

RESULTS

The cells grown out from the skin materials were fusiform and transparent fibroblasts. There were no morphologic differences between normal and acatalasemic cells. The growth rates of these normal and acatalasemic cell-lines were similar in their propagating period (from about 10th to 30th generation) as shown in Fig. 2. And most of the cell-lines became regressive from about the



Normal N_1-line: 14th and 19th generations, N_2-line: 10th and 19th generations. Acatalasemic AW-line: 10th and 18th generations.

Fig. 2 Growth Curves of Normal and Acatalasemic Cell Lines

30th generation to 40th, subsequently they proliferated again up to about the 60th generation, and then gradually grew regressive. At present, acatalasemic AT-cells, about 460 days old in the 95th generation, and normal N_2-cells, about 480 days old in the 80th generation, are barely sustaining life in our laboratory.

In normal cells the catalase activity was observed to be 314 Qo_2/mg cell protein on the average of three cell lines by the manometric method and 0.16 P. U./mg cell protein by the perborate method. In acatalasemic cells likewise minimal catalatic activity was found, e. g. 7.0 Qo_2/mg cell protein and 0.007 P. U./mg cell protein (Table 2). This minimal activity is 2.2% of normal cell activity by manometric method, 4.5% by the perborate method, and this was almost completely inhibited by 10^{-4}M sodium azide.

As shown in Tables 3 and 5, there could be recognized no significant differences between the normal and acatalasemic cells on their respiratory enzymatic activities.

Table 2 Catalatic Activity in Each Acatalasemic Cell Line

a) Manometric method. (Q_{O_2}/mg cell protein)

Normal cell lines			Acatalasemic cell lines		
Generations		Catalase activity (M±σ)	Generations		Catalatic Activity (M±σ)
N_1	15—60	316±12 (n=9)	AW	5—25	4.0±1.2 (n=9)
N_2	15—70	311±12 (n=16)	AT	9—65	7.8±0.8 (n=15)
N_3	4—45	316±11 (n=9)	AF	10—35	8.0±1.7 (n=9)
Average		314 (100.0%)	AI	8—35	8.0±2.0 (n=4)
			Average		7.0 (2.2%)*

Q_{O_2}: the volume of oxygen evolution (μl) from hydrogen peroxide for 30 minutes at 37°C.

b) Perborate method. (Perborate unit/mg cell protein)

N_3-line 0.157±0.024 P. U. /mg cell protein (n=4) (100.0%)

AI-line 0.007±0.002 P. U. /mg cell protein (n=4) (4.5%)*

Perborate Unit: number of mEq. of $NaBO_3$ decomposed at 37°C for 5 minutes.

* Relative activity expressed as per cent against the activity in normal cells.

Table 3 Succinoxidase Activity in Each Cell Line

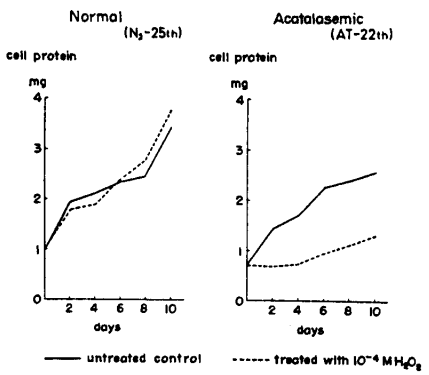
		Succinoxidase (M±σ)	Endogenous dehydrogenase (M±σ)
N_1	(n=8)	0.209±0.087	0.196±0.089
N_2	(n=8)	0.279±0.111	0.413±0.148
N_3	(n=14)	0.231±0.039	0.392±0.108
Average		0.240±0.041	0.314±0.128
AW	(n=8)	0.108±0.052	0.160±0.028
AT	(n=6)	0.147±0.052	0.301±0.028
AF	(n=5)	0.240±0.070	0.370±0.092
AI	(n=8)	0.320±0.186	0.300±0.100
Average		0.204±0.103	0.283±0.102

N_1, N_2, N_3: Normal cell lines, AW, AT, AF, AI: Acatalasemic cell lines.

When the normal cells were treated with 10^{-4}M hydrogen peroxide, the growth rate was unchanged compared with untreated control. In contrast to

this, the growth rate of acatalasemic cells became lowered as shown in Fig. 3.

When 10^{-4}M hydrogen peroxide was added onto the acatalasemic cultured cells the succinoxidase activity decreased to about 30% of the untreated control while in the normal cells its activity decreased to about 80% of the control untreated with hydrogen peroxide. As to the cytochrome oxidase activity, in acatalasemic cells the activity decreased to 60% of the control while in normal cells the activity was almost unchanged. This decrease of the succinoxidase activity in acatalasemic cells could be completely prevented by the prior addition of extracellular catalase (Tables 4 and 5). In the endogenous dehydrogenase activity which was thought to be DPNH-dehydrogenase system, no remarkable change of activity was observed in both normal and acatalasemic cells after the treatment with the hydrogen peroxide.



N_2-line: 25th generation, AT-line: 22nd generation.

Fig. 3 Growth Curves of Normal (N_2-line) and Acatalasemic (AT-line) Cells after Exposure to $10^{-4}MH_2O_2$

There were observed no remarkable differences in the growth rate and succinoxidase activity between normal and acatalasemic cells after exposure to

Table 4 Effect of Hydrogen Peroxide (10^{-4}M) on the Succinoxidase Activity in Acatalasemic Cells

Number of cases		Succinoxidase (M±σ) Untreated control	Succinoxidase (M±σ) Treated with 10^{-4}M H_2O_2	Endogenous dehydrogenase (M±σ) Untreated control	Endogenous dehydrogenase (M±σ) Treated with 10^{-4}M H_2O_2
Normal	6	0.174±0.086	0.153±0.081 (88%)*	0.165±0.058	0.142±0.035 (86%)*
Acatalasemic	5	0.110±0.053	0.030±0.018 (27%)	0.117±0.090	0.134±0.050 (82%)
Acatalasemic (Catalase Solution Added)**	3	0.105±0.067	0.105±0.054 (100%)	0.114±0.036	0.147±0.030 (102%)

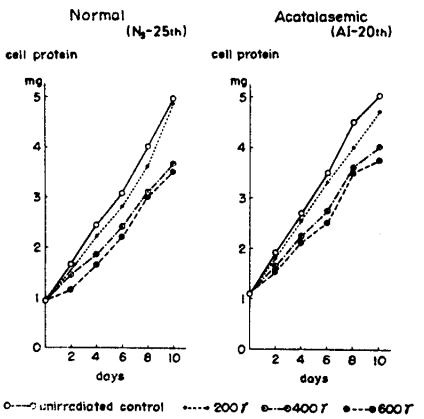
* Per cent against the activity of untreated control.

** The catalase solution added was prepared as follows: Crystalline bovine liver catalase (Wako Chemical Co.) was dissolved in 0.9% NaCl solution to 1 mg/ml and dialysed against 0.9% NaCl solution overnight. Prior to the treatment with 10^{-4}M H_2O_2, the monolayer-formed cells in bottle were overlaid with 1 ml of the above mentioned catalase solution.

Table 5 Effect of Hydrogen Peroxide (10^{-4}M) on the Cytochrome Oxidase Activity in Acatalasemic Cells

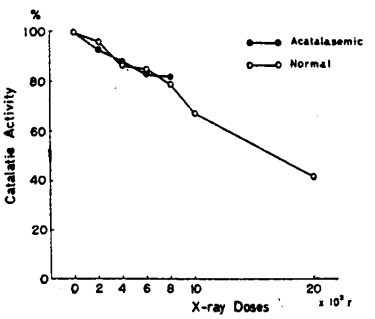
	Number of Cases	Cytochrome Oxidase Activity Untreated Control (M± σ)	Treated with 10^{-4}M H_2O_2 (M± σ)
Normal (N_3-line)	5	2.90±1.05	3.12±0.98 (107%)*
Acatalasemic (AT-line)	5	3.21±1.28	2.01±0.43 (63%)*

* Per cent against the activity of untreated control.



N_3-line: 25th generation, Al-line: 20th generation.

Fig. 4 Growth Curves of Normal (N_3-line) and Acatalasemic (Al-line) Cells after X-ray Irradiation



Catalatic activity is expressed as per cent against the unirradiated control.

Fig. 5 Effect of X-ray Irradiation on the Catalatic Activity in Acatalasemic Cultured Cells

X-ray (200 to 600 r) (Fig. 4 and Table 6). And the minimal catalatic activity in acatalasemic cultured cells decreased very slightly as well as in the case of normal cells by X-ray irradiation (Fig. 5).

DISCUSSION

Acatalasemic individuals are generally in a good health with exception that some have oral gangrene called Takahara's disease. This raises the following problems: what the physiological meaning has catalase in the human tissues and by what the catalase function is compensated in acatalasemic one. As for the above problems, TAKAHARA[1] reported that there exists 1 to 2% catalatic activity

Table 6 Effect of X-ray Irradiation on the Succinoxidase Activity in Acatalasemic Cell Lines

Cell-line	X-ray doses (r)	Number of cases	Succinoxidase (M± σ)		Endogenous dehydrogenase (M± σ)	
			a.	b.	a.	b.
N_1	200	5	0.315±0.015	0.314±0.027* (99.9%)	0.253±0.110	0.285±0.086 (112.7%)*
N_2	200	5	0.231±0.085	0.198±0.089 (85.7%)	0.351±0.080	0.368±0.084 (104.9%)
	400	4	0.196±0.036	0.189±0.063 (96.4%)	0.239±0.025	0.265±0.023 (110.9%)
N_3	200	8	0.247±0.103	0.217±0.078 (88.0%)	0.294±0.088	0.304±0.084 (103.3%)
	400	5	0.214±0.071	0.201±0.075 (93.9%)	0.328±0.052	0.328±0.097 (100.0%)
AW	200	3	0.166±0.020	0.175±0.021 (86.6%)	0.132±0.033	0.123±0.025 (93.2%)
AT	200	5	0.167±0.040	0.177±0.061 (106.0%)	0.282±0.082	0.279±0.070 (99.0%)
	400	3	0.203±0.104	0.199±0.048 (98.0%)	0.325±0.060	0.306±0.078 (94.1%)
AF	200	4	0.319±0.085	0.357±0.105 (111.9%)	0.371±0.114	0.369±0.100 (96.9%)
	400	3	0.260±0.076	0.307±0.147 (118.0%)	0.377±0.087	0.296±0.084 (80.0%)
AI	200	4	0.377±0.050	0.378±0.123 (100.0%)	0.532±0.152	0.427±0.126 (80.2%)
	400	4	0.311±0.076	0.250±0.016 (80.4%)	0.235±0.888	0.224±0.025 (95.7%)

* Per cent against the activity of unirradiated control.
a.: Unirradiated control, b.: X-ray irradiated one.

of normal blood in the acatalasemic blood by manometric method. Recently, AEBI *et al*[4] studied more precisely the minimal catalatic activity of acatalasemic blood. They separated the catalase active material, which they named 'residual catalase', from hemolysate of acatalasemic individual by Sephadex G-100 gel filtration and showed that this material was identical with normal blood catalase protein immunoelectrophoretically. In Japanese cases, OGATA *et al.*[9] likewise separated minimal catalatic substance from acatalasemic blood by gel filtration. In addition, they demonstrated that the minimal activity decomposing hydrogen peroxide, which results from the formation of methemoglobin hydrogen peroxide compound, was also found in the acatalasemic hemoglobin separated by gel filtration. Hence it is concluded that catalatic activity in acatalasemic blood reported by TAKAHARA is the sum of the activity due to the minimal catalatic substance and the catalatic action of methemoglobin-hydrogen peroxide compound.

In the author's experiment on the culured cells of the skin from acatalase-

mic individuals, an existence of a slight catalatic activity was found by manometric method as well as FEINSTEIN's method. Since these cultured cells do not contain hemoglobin at all, this catalatic activity is not the one from the methemoglobin found in the acatalasemic blood.

As to the susceptability of acatalasemic cells to extrinsic hydrogen peroxide, the growth rate of the cells was significantly disturbed and the activity of respiratory enzymes in the cells was markedly diminished by adding 10^{-4}M hydrogen peroxide. From these results the resistance of the acatalasemic cells to extrinsic hydrogen peroxide can be said to be very low. These findings agree with those of DOI's experiment[10] in which he successfully developed oral gangrene of ducks, originally acatalasemic, by the submucous injection of hydrogen peroxide.

Therefore, it seems to be clear that when the acatalasemic cells are exposed to highly concentrated extrinsic hydrogen peroxide (as in the case of bacterial proliferation), the minimal catalatic substance turns to be not enough to decompose hydrogen peroxide and respiratory enzymes are inactivated by the extrinsic hydrogen peroxide, resulting in tissue necrosis called Takahara's disease.

In contrast to the effects of the extrinsic hydrogen peroxide, there could be recognized no differences between acatalasemic and normal cells on the sensitivity to X-ray irradiation. According to AEBI[11] the hydrogen peroxide produced by X-ray irradiation is 3.2 to 3.9 μM per liter per 1,000 r doses. Therefore, in the present experiment it is assumed that the minimal catalatic substance can decompose such a small amount of hydrogen peroxide produced by irradiation (200 to 600 r). However, it is premature to make any definite conclusion from these results alone.

SUMMARY

For the purpose to clarify whether minimal catalatic activity exists in Japanese acatalasemic cells or not and the manner how extrinsic hydrogen peroxide affects the acatalasemic cells, the author performed tissue cultures using the skin specimens from four acatalasemic persons affected with Takahara's disease and studied the nature of these cultured cells. The results are summarized as follows.

1. Between normal and acatalasemic cultured cells, no morphological differences could be seen and the growth rate of these cell-lines was similar to one another.

2. On the activity of succinoxidase and cytochrome oxidase there could be observed no difference between normal and acatalasemic cells.

3. In each acatalasemic cell line the minimal catalatic activity was observed and it seemed that this activity has an important role in decomposing hydrogen

peroxide under normal metabolic pathway.

4. After treating with 10^{-4}M hydrogen peroxide, respiratory enzyme activities and the growth rate in the acatalasemic cells were markedly disturbed, while in normal cells these remained almost intact.

5. There could be observed no differences between normal and acatalasemic cultured cells after X-ray irradiation (200 to 600 r) on the succinoxidase activity, catalatic activity and growth rate.

ACKNOWLEDGEMENT

The author wishes to thank Professor M. OGATA and Professor S. TAKAHARA of our Medical School for their helpful advices, and to Dr. OHKURA (Associate Professor, Dept. of Human Genetics, Tokyo Medical and Dental University) and Dr. SHIROYAMA (Professor, Dept. of Oral Surgery, Osaka Dental University) for their supplying the skin specimens. Thanks are also due to Professor T. ODA for the use of oxymeter and to Mr. Y. KURODA for his technical assistance in tissue culture.

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