54. Hideo Sugimoto and Michiko Aoshima*¹ : Pattern of Enzyme Activity of the Nitrogen Mustard Sensitive and the Resistant Ascites Tumors.

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Rapid acquisition of high resistance of Yoshida sarcoma to an alkylating agent by a short time contact *in vitro* with this agent is a problem of great concern in the investigation of cancer chemotherapy, on that Sakurai, *et al.*¹⁾ discussed in detail in the recent publication.

By repetition of this procedure, a line of Yoshida sarcoma, 10,000-fold resistant to the original line, was established, which was found however not to be different in its cytomorphological or pathological features from its original line, except that the growth rate of the former in tissue culture seemed to be slightly less than that of the latter. Therefore it was presumed that the quite difference in susceptibility of the resistant line to the agent from that of the original may due either to the variance of membrane permeability of the cell or subcellular particles, or to the partial shift of some metabolic pathways, the former problem of which was discussed recently by one of the present authors.²⁾

For the purpose of investigation on the latter question, this paper deals with the determination of enzyme activity of the resistant and the original Yoshida sarcoma. Besides, the enzyme activity of the rat ascites hepatomas induced by an azo dye in the Medical Institute of the Sasaki Foundation were also presented in this paper. Some of these hepatomas are inherently resistant to alkylating agents and the others are not, in spite of the fact that all of these tumors were established by the same carcinogen and same procedure with the same strain of animal. It was therefore an interest of the author to compare the patterns of enzyme activities of the acquired and the inherently resistant tumors.

Materials and Methods

All the tumors used were kept in the peritoneal cavity of Donryu rat by successive transplantation. In most cases, the animals bearing tumor were sacrificed between the 5th and 14th day after inoculation, when the tumor reached to maximum growth.

The tumor infiltration on the omentum was then excised, promptly washed with cold physiological saline, pressed softly between two sheets of filterpaper. The weighed tumor mass was cut to small pieces and mashed with thrice quantity of dist. H₂O using a teflon pestle homogenizer. The homogenate was diluted with the adequate buffers or dist. H₂O if necessary according to the necessity of each procedure of determination. The infiltration on the omentum was usually consisted of the homogenous mass of the tumor cells without accompanying any necrosis and therefore its use for such biochemical experiments is generally recommended.

Tumor Strains—The resistant line of Yoshida sarcoma used in this experiment was RA_{C-21}, which was established by Sakurai and Moriwaki¹⁾ by twenty one successive contacts *in vitro* of the Yoshida sarcoma cells with a dilute solution of methyl bis(2-chloroethyl)amine hydrochloride (HN₂). The resistance index of this line reached to 10,000 times of the original. The tumor strains denoted by AH were the rat ascites hepatomas induced by azo dye in the Medical Institute of the Sasaki Foundation, of which AH13, 130, and 99 were found to be sensitive to HN₂, AH7974, and 311 strongly resistant, and AH49 moderately resistant. The tumor strains of PD-series, seen in Table I, VIII, were also azo dye-induced rat ascites hepatomas, of which sensitivity to HN₂ has not been determined yet.

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Determination of Protein Content of the Tissues—Protein was determined by the method described by Lowry³⁾ using Folin-Ciocalteu reagent.

Acid and Alkaline Phosphatase—Acetone powder of the tumors obtained from 5 or 6 tumor bearing rats was used for determination. The powder was added with dist. H₂O and centrifuged (2,000 r.p.m.) at 5° for 10 min. The supernatant was assayed for its enzyme activity by the method described by Ohmori.⁴⁾ The activity was denoted by mmole of hydrolyzed substrate per mg. of protein in the supernatant per hr.

Choline Esterase—The homogenate of the tumor tissue (10% in pH 7.3 phosphate buffer) was centrifuged at 3,000 r.p.m. for 5 min. One ml. of the supernatant was determined for the enzyme activity by the method described by Hesterin.⁵⁾ The activity was expressed by mmole of hydrolyzed substrate per mg. of protein in the supernatant per hr.

β -Glucuronidase—The enzyme solution was prepared by centrifugation of the homogenate (25%) of the tumor tissue. The supernatant was diluted to the adequate concentration by acetate buffer. The determination was carried out by the method described by Talalay, Fishman, and Huggins.⁶⁾ and the activity was expressed by an unit which denoted μ g. of hydrolyzed substrate per g. of the wet tissue per hr.

Sulfatase—The homogenate (25%) of the tissue was determined for the enzyme activity by the method described by Roy.⁷⁾ The activity was expressed by μ mole of the hydrolyzed substrate per g. wet tissue per hr.

Asparaginase—The homogenate (25%) was used for the enzyme reaction by the method described by Saitoo and Meister, *et al.*⁸⁾ Determination of the liberated NH₃ was carried by the method of Conway, *et al.* and Hatano.⁹⁾ The activity was expressed by mg. of N₂ of NH₃ liberated per ml. of the homogenate (25%) per hr.

Glutaminase—Determination was carried out by the method described by Gilbert, *et al.*¹⁰⁾ The method and indication of the activity were almost same as those of asparaginase determination.

Glucose 6-Phosphatase—With the homogenate (25%), the enzyme activity was assayed by the method described by Campbell.¹¹⁾ Liberated inorganic phosphate was determined by the method reported by Takahashi.¹²⁾ The activity was denoted by mg. of H₃PO₄ per g. wet tissue per 30 min.

Glutamic-Pyruvic-Transaminase (G. P. T.)—With the homogenate (25%), the enzyme activity was determined by the method described by Wroblewski, *et al.*¹³⁾ The activity was indicated by mmole of pyruvic acid per g. wet tissue per hr.

Glutamic-Oxaloacetic-Transaminase (G. O. T.)—With the homogenate (25%), the enzyme activity was assayed by the method reported by Cabaud.¹⁴⁾ The activity was indicated by mmole of oxaloacetic acid per g. wet tissue per hr.

Catalase—With the homogenate (5%), the enzyme activity was determined by the method published by Euler and Josephson.¹⁵⁾ The activity was indicated by Kat. f., but, in this experiment, the wet tissue weight was used for calculation instead of the dry weight.

Pyruvic Dehydrogenase—The enzyme activity was determined manometrically with the homogenate (25%) by the method reported by Silverman, *et al.*¹⁶⁾ The activity was denoted by μ mole of CO₂ per g. wet tissue per hr.

Xanthine Oxidase—The enzyme activity was measured with the homogenate (25%) by the method described by Dhungat, *et al.*¹⁷⁾ The activity was indicated by μ l. of O₂ per g. wet tissue per 2 hr.

Cytochrome C Oxidase—The enzyme activity was measured with the homogenate (25%) by the method described by Straus.¹⁸⁾ The activity was expressed by indophenol blue unit per g. wet tissue per hr.

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Lactic Dehydrogenase—The enzyme activity was assayed with the homogenate (25%) by the method reported by Cabaud, *et al.*¹⁹⁾ However, lactic acid, instead of pyruvic acid, was determined in this experiment by the method described by Barker and Summerson.²⁰⁾ The activity was indicated by mg. of lactic acid per g. wet tissue per 10 min.

Phosphorylase—The enzyme activity was measured with the homogenate (25%) by the method described by Akabori.²¹⁾ H_3PO_4 liberated from glucose 1-phosphate was determined by the method described by Takahashi.¹²⁾ The activity was denoted by $\mu g.$ of H_3PO_4 per g. wet tissue per 15 min.

Hexokinase—The enzyme activity was determined with the homogenate (25%) by the method reported by Hoare and Kerly.²²⁾ Glucose and fructose were measured by Somogyi-Nelson's method. The activity was indicated by mg. of decreased sugar per g. wet tissue per 10 min.

Results and Discussion

The specific activity of the hydrolyzing enzymes of the tumors were demonstrated in Table I~VII. The activities of all the enzymes except phosphatase were found to be far more deficient when compared with those of the normal rat liver. In case of alkaline phosphatase, a very high activity was observed with a few kinds of the hepatomas, while no difference in the activity of the same enzyme was existing between two lines of Yoshida sarcome, the resistant and the original.

Such a difference between these two lines of Yoshida sarcoma was found only in case of sulfatase as shown in Table IV. The similar deficiency of activity in the tumor strains was also seen with asparaginase and glutaminase, but grade of deficiency of the latter enzyme seemed to be slighter than that of the former. Besides, a variety of grade of activity of glutaminase was observed with variety of the strains of the tumors. A hepatoma, AH 7974, was found to have a comparatively high activity of cholinesterase, that seemed to be interesting because this hepatoma has been known to be inherently resistant to nitrogen mustard on the one hand, and this enzyme is very sensitive to an attack of alkylating agent on the other.

In some cases of cancer patients, a remarkable increase of β -glucuronidase activity in the serum or stomach juice has been claimed but the activity of the same enzyme of above-mentioned experimental tumors was proved unexpectedly to be far lower than that of the normal liver, as indicated in Table III.

Regarding the activity of transaminases and catalase, the results of determination were demonstrated in Table VIII~X, in which no difference between two lines of Yoshida sarcoma was confirmed. Moreover, deficiency of the activities of these enzymes seemed to be very striking.

TABLE I. Acid and Alkaline Phosphatase Activity of Omentum Infiltration (Acetone Powder)

Strain	mmole unit/mg./hr.		Strain	mmole unit/mg./hr.	
	Acid	Alkaline		Acid	Alkaline
(Normal liver)	3.5	0.5	PD 31	5.2	1.5
Y. sar.	4.2	0.7	PD 107	4.9	3.5
Resistant Y. sar.	3.6	0.8	PD 58B	5.5	3.2
AH 130	5.8	1.8	PD 38	5.0	1.2
AH 7974	3.0	1.4	PD 63	5.1	0.9
PD 58C	7.3	1.7	PD 68C	6.2	3.6
PD 100	6.4	1.1	PD 45	6.1	1.8
PD 114	5.6	2.7	PD 51	5.5	1.0
PD 37	6.4	0.9	PD 49	5.1	0.7

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TABLE II. Cholinesterase Activity of Omentum Infiltration

Strain	No. of determinations	mmole/mg./hr.	
		Average	Range
(Normal liver)	5	0.38	0.27~0.48
Y. sar.	5	0.12	0.01~0.30
Resistant Y. sar.	4	0.09	0.03~0.13
AH 130	6	0.21	0.09~0.39
AH 7974	5	1.21	0.94~1.50

TABLE III. β -Glucuronidase Activity of Omentum Infiltration

Strain	No. of determinations	Phenolphthalein unit/g./hr.	
		Average	Range
(Normal liver)	5	34,400	31,200~38,000
Y. sar.	6	11,780	8,300~13,500
Resistant Y. sar.	6	10,020	6,100~14,000
AH 130	4	5,200	3,000~6,400
AH 7974	5	5,350	3,000~6,900
AH 49	6	7,150	3,200~10,000
AH 99	6	6,180	3,800~11,000
AH 311	3	4,300	2,500~5,500

TABLE IV. Sulfatase Activity of Omentum Infiltration

Strain	No. of determinations	<i>p</i> -Nitrophenol μ mole/g./hr.	
		Average	Range
(Normal liver)	6	80.6	71.3~90.0
Y. sar.	3	14.9	13.4~16.2
Resistant Y. sar.	3	29.2	26.0~34.6
AH 130	2	25.0	24.5~25.5
AH 7974	3	14.6	14.0~15.0
AH 49	3	13.8	12.8~14.3
AH 99	3	17.8	13.1~26.3

TABLE V. Asparaginase Activity of Omentum Infiltration

Strain	No. of determinations	NH ₃ -N mg./ml./hr.	
		Average	Range
(Normal liver)	5	27.0	23.5 ~ 34.0
Y. sar.	4	0.32	0.10~ 0.57
Resistant Y. sar.	3	0.18	0.10~ 0.30
AH 130	3	0.43	0.35~ 0.54
AH 7974	5	3.7	3.6 ~ 4.6
AH 49	3	0.23	0.15~ 0.35
AH 99	5	1.8	1.0 ~ 3.0
AH 311	3	3.1	2.3 ~ 4.2

TABLE VI. Glutaminase Activity of Omentum Infiltration

Strain	No. of determinations	NH ₃ -N mg./ml./hr.	
		Average	Range
(Normal liver)	5	31.9	27.6~35.0
Y. sar.	3	8.2	6.0~12.3
Resistant Y. sar.	3	14.3	12.5~16.3
AH 130	3	1.2	0.7~1.5
AH 7974	4	8.4	4.3~13.3
AH 49	5	0.7	0.2~0.9
AH 99	4	0.8	0.7~1.0
AH 311	3	2.8	2.3~3.0

TABLE VII. Glucose 6-Phosphatase Activity of Omentum Infiltration

Strain	No. of determinations	Pi mg./g./30 min.	
		Average	Range
(Normal liver)	5	9.2	9.0~10.4
Y. sar.	4	0.1	0.0~0.2
Resistant Y. sar.	4	0.2	0.0~0.5
AH 130	1	0.3	
AH 7974	2	0.3	0.1~0.4
AH 49	2	0.2	0.2
AH 99	2	0.1	0.1

TABLE VIII. Glutamic-Pyruvic-Transaminase Activity of Omentum Infiltration (Acetone powder)

Strain	mmole/g./hr.	Strain	mmole/g./hr.
(Normal liver)	4.6	PD 58C	0.3
Y. sar.	0.8	PD 96	0.2
Resistant Y. sar.	1.4	PD 100	0.2
PD 58B	0.4	PD 107	0.3

TABLE IX. Glutamic-Oxaloacetic-Transaminase Activity of Omentum Infiltration

Strain	No. of determinations	mmole/g./hr.	
		Average	Range
(Normal liver)	4	12.2	10.5~14.5
AH 130	4	0.83	0.43~1.05
AH 7974	3	1.11	0.85~1.50
AH 66F	3	1.67	1.50~1.88

TABLE X. Catalase Activity of Omentum Infiltration

Strain	No. of determinations	Kat. f/g.	
		Average	Range
(Normal liver)	20	19.0	11.0~32.0
Y. sar.	2	0.4	0.4
Resistant Y. sar.	2	0.2	0.1~0.3
AH 130	2	0.3	0.3
AH 7974	2	0.4	0.4
AH 49	2	0.3	0.2~0.3
AH 99	2	0.3	0.3
AH 311	2	0.2	0.2
AH 322	2	0.2	0.1~0.2
AH 57B	2	0.4	0.3~0.4
AH 122B	2	0.5	0.4~0.5

TABLE XI. Pyruvic Dehydrogenase Activity of Omentum Infiltration

Strain	No. of determinations	CO ₂ μ mole/g./hr.	
		Average	Range
(Normal liver)	5	41.4	32.9~51.7
Y. sar.	4	19.4	18.1~21.1
Resistant Y. sar.	5	17.4	13.9~19.5
AH 130	4	16.7	12.9~24.0
AH 7974	2	15.9	15.4~16.4
AH 49	5	24.0	13.8~33.0
AH 99	4	20.2	18.5~22.6
AH 311	3	19.8	19.0~21.4

TABLE XII. Xanthine Oxidase Activity of Omentum Infiltration

Strain	No. of determinations	O ₂ Uptake μ l./g./2 hr.	
		Average	Range
Y. sar.	4	109	92~116
Resistant Y. sar.	6	117	82~148
AH 130	4	284	211~356
AH 7974	7	327	126~535
AH 49	5	281	176~520
AH 99	3	307	164~504
AH 311	4	221	146~266

TABLE XIII. Xanthine Oxidase Activity of Tumor Bearing Rat Liver

Strain	No. of determinations	O ₂ Uptake μ l./g./2 hr.	
		Average	Range
Normal	5	1421	1137~1923
Resistant Y. sar.	6	1160	1045~1396
AH 130	3	1083	933~1346
AH 7974	5	805	785~1259
AH 49	5	1184	945~1477
AH 99	3	1394	1182~1557

TABLE XIV. Cytochrome C Oxidase Activity of Omentum Infiltration

Strain	No. of determinations	Indophenol blue unit/g./hr.	
		Average	Range
(Normal liver)	5	68.8	60.3~76.8
Y. sar.	5	12.1	10.7~12.9
Resistant Y. sar.	4	9.8	7.9~10.7
AH 130	5	9.9	8.7~10.7
AH 7974	5	7.2	4.9~9.0
AH 49	5	7.5	6.9~8.0
AH 99	5	8.4	7.7~9.0
AH 311	3	11.9	8.6~16.5

TABLE XV. Lactic Dehydrogenase Activity of Omentum Infiltration

Strain	No. of determinations	Lactic acid mg./g./10 min.	
		Average	Range
(Normal liver)	6	444	396~470
Y. sar.	7	228	183~255
Resistant Y. sar.	5	300	242~350
AH 130	5	174	132~204
AH 7974	3	167	138~186
AH 49	7	198	162~223
AH 99	3	112	91~124
AH 311	3	80	59~97

TABLE XVI. Phosphorylase Activity of Omentum Infiltration

Strain	No. of determinations	Pi μ g./g./15 min.	
		Average	Range
(Normal liver)	5	4010	3680~4180
Y. sar.	3	694	624~865
Resistant Y. sar.	3	629	610~653
AH 130	4	593	508~725
AH 7974	4	601	550~624
AH 49	5	731	710~754
AH 99	3	662	565~740
AH 311	3	555	362~652

TABLE XVII. Hexokinase Activity of Omentum Infiltration (Substrate : Glucose)

Strain	No. of determinations	Decreased glucose mg./g./10 min.	
		Average	Range
(Normal liver)	6	(trace)	
Y. sar.	3	7.1	6.7~7.6
Resistant Y. sar.	3	5.7	5.3~6.1
AH 130	6	6.0	4.1~7.9
AH 7974	7	6.1	4.4~10.5
AH 49	7	9.3	7.9~11.0
AH 99	4	4.9	4.1~5.9
AH 311	2	3.6	3.2~3.9

TABLE XVIII. Hexokinase Activity of Omentum Infiltration (Substrate : Fructose)

Strain	No. of determinations	Decreased fructose mg./g./10 min.	
		Average	Range
(Normal liver)	3	(trace)	
Y. sar.	2	"	
AH 130	2	1.3	1.0~1.6
AH 7974	2	(trace)	
AH 49	2	2.8	2.5~3.1
AH 99	2	1.1	0.9~1.3
AH 311	2	0.3	0.1~0.3

The data with dehydrogenases and oxidases were shown in Table XI~XV, in which the same result was also obtained with the two lines of Yoshida sarcoma. The activity of the tumors were remarkably smaller in general than that of the normal liver, but, regarding lactic dehydrogenase alone, activity differed with the strains of tumors. Table XIII indicated the activity of xanthine oxidase of the host liver, which seemed not to be affected even at the maximum growth of tumor, with an exception of AH7974.

It has been found recently by Sakurai, Tashiro, and Aoshima²³⁾ that the content of glycogen and oligosaccharides, a principal member of which was determined to be maltose, was highly elevated in the cells of resistant line of Yoshida sarcoma. However, as seen in Table XVI~XVIII, any possible comprehension of this phenomenon was not deduced from this pattern of activities of phosphorylase and hexokinase. Besides, by this method of determination, activity of hexokinase of the normal liver appeared to be almost null.

In summarizing these results, it was not successful to explain a possible mechanism of an acquired resistant of Yoshida sarcoma by the metabolic feature of the cells from the view of enzyme activities. In this regard, only difference was observed in the activity of sulfatase, the meaning of which however is still in question.

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Summary

The paper deals with a comparison of the pattern of activity of enzymes of the original Yoshida sarcoma with that of a line of the same tumor, which is 10,000-fold resistant to HN_2 . Only difference so far obtained by these experiments with sixteen kinds of enzymes was found in the activity of sulfatase. Patterns of enzyme activity of several rat ascites hepatomas were also presented, some of which are inherently resistant to HN_2 , some are very sensitive, and the rest of them moderately sensitive.

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23) unpublished