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Transaminases of Hepatic Tissue Culture Cells and the Effect of Carbon Tetrachloride on Their Leakage[1]

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The direct effect of CCl_4 on leakage of cellular glutamic oxaloacetic and glutamic pyruvic transaminases of a line of hepatic tissue culture cell, HTC cell, into medium was quantitatively studied. Cellular levels of soluble isozymes of the both aminotransferases were considerably lowered by addition of CCl_4 to culture medium at a concentration of 10 mM followed by 48 and 96 hr of culture. Contrasting increase or less influenced decrease of enzyme activities in bathing fluid after treatment made the leaking indexes of the enzymes(cellular secretion of activities found in medium/cellular activities) twice or more as large as control depending on the concentrations of CCl_4 in medium and lengths of treatment. Simulating study on change of CCl_4 concentration in bathing fluid with time of culture showed a rapid and extensive fall of it upon the addition of CCl_4 with a later stable phase probably in equilibration with the agent in the gas phase within culture flask.

Keywords——transaminase; liver cell; tissue culture; carbon tetrachloride; cell membrane; permeability

Cytotoxic agents often have a non-specific increasing effect on the permeability of cell membrane to many of cellular components. An example of such an undesirable effect on the cell is increased leaking of cellular transaminases, glutamic oxaloacetic transaminase (GOT, EC 2.6.1.1) and glutamic pyruvic transaminase(GPT, EC 2.6.1.2), into circulation from the liver following administration of CCl_4 to animals such as the rat [3,4] and dog.[5,6]

It is reported, on the other hand, that administration of CCl_4 to rats stimulates the sympathetic nervous system to result in a constriction of the blood vessels with a consequent depletion of blood supply to the liver in coincidence with occurrence of hepatic necrosis.[7] Therefore various hepatic damages such as increased permeability of cell membrane and abnormal lipid metabolism following CCl_4 administration might be an indirect effect mediated by excitation of autonomic nervous system by the drug.

The method of tissue culture of liver cells must be reliable to determine whether the effect of CCl_4 on leakage of the enzymes out of the liver can be direct one on the respective cell or indirect one mediated by any effect of the agent on other related tissue cells.

In the present work mitochondrial and soluble GOT's and GPT's were identified in the cultured hepatic cell, hepatic tissue culture(HTC) cell, and the direct effect of CCl_4 on the

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cell was studied quantitatively in terms of the leakage of the both transaminases out of the cells in relation to the concentrations of CCl_4 in bathing fluid and periods of treatment.

Materials and Methods

Cell——The HTC cell, originated in a rat hepatoma(Morris hepatoma) and established as a cell line by Thompson and colleagues,[8] was obtained by courtesy of Dr. M. Shikita at the National Institute of Radiological Sciences, Chiba-shi, Japan. The cells were grown as monolayers on the glass of flat flask(250 ml of capacity each) in the slightly modified 858 medium[7] supplemented with 10%(v/v) of inactivated bovine serum. The flasks were filled with air and stoppered throughout cultivation. Trypsinization was applied to the confluent layers of cells for subcultivation.

Chemicals——Commercial compounds of special grade were used without further purification for all agents but aniline and diphenylamine in GOT and deoxyribonucleic acid (DNA) assays, respectively. Special grade aniline (Wako Pure Chemicals Company, Japan) was distilled under aspiration after dehydration and stored under N_2 gas. Diphenylamine was recrystallized from *n*-hexane. Diethylaminoethyl (DEAE) cellulose (Brown Company, New York, N.Y., U.S.A.; Capacity: 0.86 meq./g) was activated with 0.1 N HCl and 0.1 N NaOH sequentially before use.

Enzyme Separation——For each sample of enzyme assay confluent layers of cells of 8—12 flasks were combined into about 100 ml of phosphate-buffered saline (PBS), pH 7.4, containing 8.0 g of NaCl, 0.2 g of KCl, 2.9 g of $Na_2HPO_4 12H_2O$, 0.2 g of KH_2PO_4 and 0.2 g of D-glucose per liter after washing the cell layers with the same salt solution, separated by centrifugation, homogenized with 7 ml of 0.34 M sucrose solution, subjected to repeated freezing in a dry ice-acetone bath and then thawing, and fractionated by a centrifugation at $105000 \times g$ for 60 min to obtain the supernatant fraction. The supernatant fraction was dialysed in Visking tube to 0.001 M potassium phosphate buffer solution (pH 7.5) or to 0.005 M potassium phosphate buffer solution (pH 7.0) for samples of GOT and GPT separations, respectively, for 9 hr. The dialysate, concentrated up to about 2 ml with the use of polyethylene glycol as a water absorbent at 4 °, was applied on a column (1.5×15 cm) of DEAE cellulose previously equilibrated with 0.001 M potassium phosphate buffer solution or one (1.2×10 cm) previously equilibrated with 0.005 M potassium phosphate buffer for GOT and GPT separations, respectively. The salt concentration of the eluants was stepwise increased up to 0.05 M or 0.2 M for the two kinds of transaminase as specified in the results. The elution profiles of mitochondrial and soluble forms of the two kinds of transaminase of HTC cell were confirmed by the comparable performance of column chromatography of the two kinds of supernatant fraction of the homogenate of rat liver with 0.34 M sucrose: one(a) was obtained by direct centrifugation at $105000 \times g$ of the homogenate and the other(b) obtained by centrifugation at $9000 \times g$ of the mitochondrial fraction after twice repeated freezing and thawing of it. The fraction (b) would be expected to contain the mitochondrial form exclusively and (a) to contain both of the two forms, but mainly the soluble form (data not presented.)

Enzyme Assay——An aliquot, 0.2 g or 0.5 ml, of each eluted fraction (2—3 ml each) was subjected to enzyme assay according to the method of Wada and Snell.[10] GPT activity was shown by the amount (μ moles) of pyruvate produced per hr per ml of fraction and GOT activity by the amount of oxaloacetate, which was actually converted to pyruvate with reduction by aniline before hydrazone formation. The sum of activity of soluble enzymes separated into fractions of the column chromatography was calculated into the activity per mg DNA of original cells for each enzyme as accumulation of the enzymes within cells in the experiments of CCl_4 treatment. The enzyme activities found in culture medium were assayed using 0.2—1.0 ml each of medium samples taken in the end of culture without column separation by the same procedure as that for the eluted samples above.

DNA Determination of Cells——DNA of the sediments obtained by the centrifugation at $105000 \times g$ of the cell homogenates was extracted into a solution of trichloroacetic acid (5%) and determined by the method of Dische.[11]

Protein Determination of Column Eluates——0.2 or 0.5 ml of each eluated fraction was diluted with 2 N NaOH solution up to 1.0 ml and subjected to protein determination according to the method of Lowry, *et al.*,[12] with bovine serum albumin as standard.

Treatment with CCl_4 of Cells——CCl_4 was mixed in ethanol (0.2 or 2.0 M CCl_4) and added to each flask to make its final concentration in medium 1 or 10 mM at the time of addition. The cells were incubated for 48 hr under such conditions with closed air phase. CCl_4 was added newly at the renewal of medium and

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incubation was performed for another 48 hr in the groups of 96 hr treatment. Control cells were exposed to ethanol (finally 0.5%) only.

Determination of CCl_4 in Medium by a Gas-Liquid Chromatography——The concentration of CCl_4 in medium changed with time of incubation because of its volatility. Information of time courses of the change of CCl_4 concentration, initially 1 and 10 mM, was obtained by determination of its concentration finally in 48 hr of incubation of cells in each experiment and also by cheking its concentration in water in simulating systems using the same flasks as the culture with successive sampling, at 0.5, 3, 8, 13, 24 and 48 hr, after addition of CCl_4. Samples for the gas-liquid chromatography were prepared by extraction of 15.0 ml each of medium or water with 0.5 ml of carbon disulfide and 1.0 μl of each was subject to analysis by an apparatus of Hitachi 063 under the following conditions; detector: FID (H_2=0.6 kg/cm^2, air=1.2 kg/cm^2) at 95°; carrier gas: N_2 (30 ml/min); column: stainless steel tube (1 m) at 40°; stationary phase: polyethylene glycol 1500, 60/80 mesh.

Results

Isozymes of the Transaminases of HTC Cell

Figure 1 and 2 represent the results of chromatography for the separation on DEAE cellulose column of each isozyme of the transaminases, soluble and mitochondrial GOT's and GPT's, in the supernatant fractions obtained by centrifugation of the cell homogenates at 105000×***g***. The supernatant fraction was found to be highly possessed of GOT activity compared to GPT activity. Contaminating mitochondrial GOT was eluted by 0.001 M phosphate, and a prominent bend of soluble one by 0.05 M phosphate(Fig. 1). A little mitochondrial GPT activity appeared in 0.2 M phosphate fractions, while a concentrated activity of soluble GPT was eluted in 0.05 M phosphate(Fig. 2).

Most of the mitochondrial enzymes was retained in the sediments of centrifugation of the cell homogenates (data not shown). Soluble and mitochondrial GOT's and GPT's of HTC cell were identified by comparing with elution profile on DEAE cellulose column of each of those isozymes in the soluble and mitochondrial fractions of the rat liver homogenate.

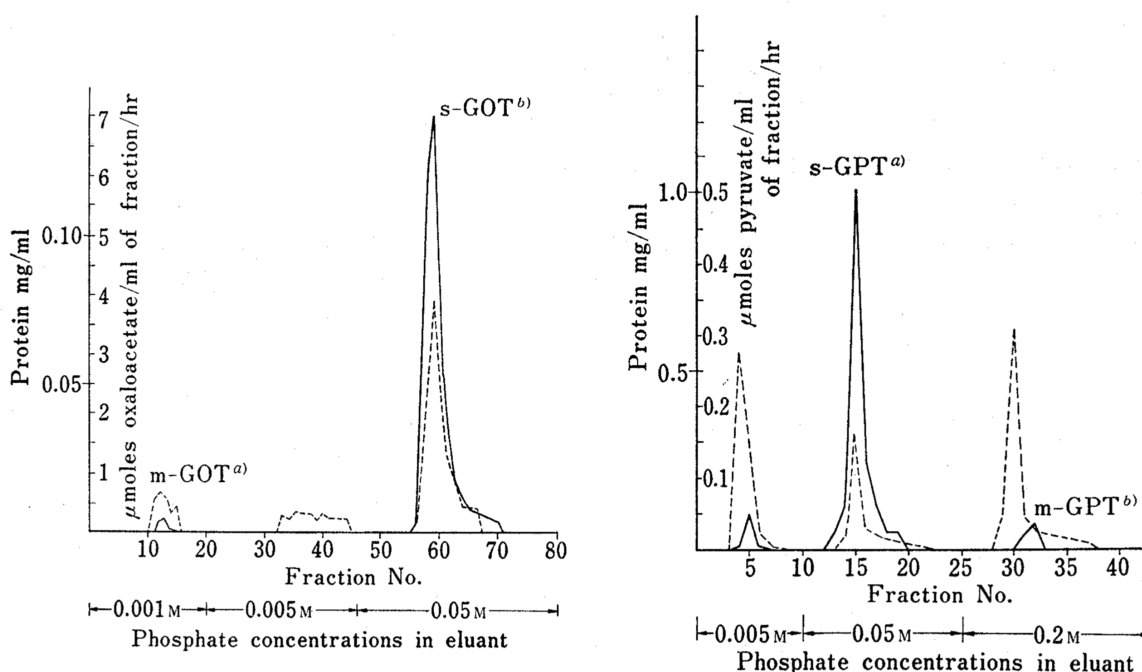

Fig. 1. DEAE Cellulose Column Chromatography of GOT' s of HTC Cells (Supernatant Fraction)

a) Mitochondrial GOT, *b)* Soluble GOT
——: GOT activity, -----: protein

Fig 2. DEAE Cellulose Column Chromatography of GPT' s of HTC Cells (Supernatant Fraction)

a) Soluble GPT, *b)* Mitochondrial GPT
——: GPT activity, -----: protein

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Effect of CCl_4 Treatment on Leakage of the Transaminases out of Cells

The two transaminases were found normally to be secreted into medium (see both transaminase activities in medium for control cultures in Table I). Treatment with CCl_4 of the cell for 48 hr or more at an added concentration of 10 mM enhanced greatly the leakage of both enzymes from the cell into medium(Expt. 1, 3 and 6 in Table I). It is noted that enhancement of leaking of GPT was much larger than that of GOT at the same condition of treatment (Expt. 3 *vs.* 6). Treatment with CCl_4 for 96 hr at an added concentration of 1 mM with renewing the medium at 48 hr also enhanced the leakage of GOT from the cell (Expt. 2).

TABLE I. Effect of CCl_4 on Accumulation and Leakage of GOT and GPT in HTC Cells

Enzyme	Exp. No.	CCl_4 treatment			Transaminase activity following treatment (μ mol. products[b]/hr)		Leaking index	
		Addition (mM)	Duration (hr)	Harvested DNA[a] (μg)	Cell[c] (per mg DNA) (A)	Medium (per mg cell. DNA) (B)	(B)/(A)	Ratio to control
GOT	1	10	96[d]	196	7.1	219.0	31.0	15
		0		844	71.7	147.9	2.06	
	2	1	96[d]	486	70.2	198.3	2.82	1.9
		0		539	100.8	151.7	1.51	
	3	10	48	612	77.2	531.0	6.88	1.9
		0		698	86.6	308.2	3.56	
	4	1	48	700	124.9	298.2	2.39	0.9
		0		737	105.1	286.7	2.73	
	5	1	48	834	95.9	270.8	2.82	1.1
		0		735	117.2	307.6	2.63	
GPT	6	10	48	1058	0.823	11.56	14.1	4.8
		0		1314	6.924	20.44	2.9	

a) obtained from 8—12 bottles of cells for each group
b) oxaloacetate for GOT and pyruvate for GPT
c) Activity of the soluble enzyme, GOT or GPT, in the supernatant fraction is shown.
d) Culture medium was renewed at 48 hr of treatment with the same one as preceding.

The effect of CCl_4 on leaking of those enzymes is probably only due to increase of permeability of the cell membrane to the enzymes and not to promotion of synthesis or cellular accumulation of them, because, in spite of the fact that the cellular accumulation of the enzymes (shown in column (A) of Table I) was unchanged or even lowered by treatment with CCl_4, secretion of the enzymes into medium(represented by the values shown in column (B)) was either increased(as in the experiments of No. 1, 2 and 3) or not so influenced as was the cellular retention(as in the experiment for GPT).

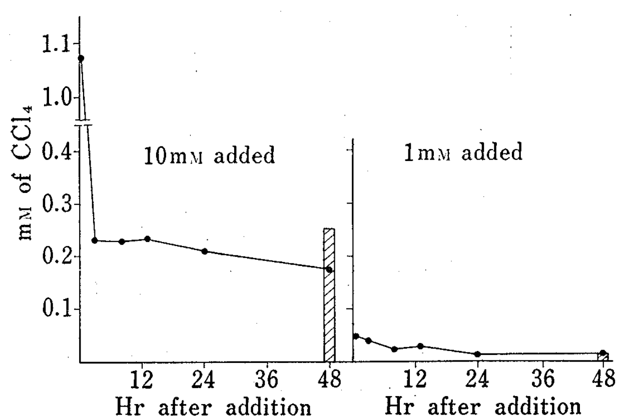

Fig. 3. Concentration Change with Time of CCl_4 in Water and Medium Contained in Culture Flasks at 37°

——: water, ▨: medium with cells

The activity of the enzymes in medium was generally so little that it could not be assayed after separation by column chromatography. Therefore their origin in the cellular compartment was not defined. Evidence has been reported for the secreted GOT from the liver cell

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in nomal rats as well as CCl_4-intoxicated rats to originate mainly in the cytosol of the cells.[3]

Equilibrated Concentration of CCl_4 in Incubation Medium

It was found in the model systems using water enclosed in the culture bottles and by determination of the final concentration in the used medium in the culture experiments that the concentration of CCl_4 in the fluid phase decreased very rapidly and considerably upon addition; about 1/20 and 1/10 of addition at the time as early as 30 min after addition, 1/25 and 1/50 at 3 hr, in added concentrations of 1 and 10 mM, respectively, and about 1/50 commonly at 48 hr(Fig. 3). Therefore it should be assumed that exposure of cells to CCl_4 was actually performed at about 0.2 and 0.02 mM for most part of incubation time in added concentrations of 10 and 1 mM, respectively, in the present series of experiment.

Discussion

It was shown in the present experiments that treatment of HTC cells with about 0.2 mM of CCl_4 in culture medium for no more than 48 hr caused an increase of leakage of cellular GOT and GPT from the cells. Many reports[13] have shown in rats and other animals that administration of CCl_4 in doses of 0.2—1.5 ml/kg of body weight to animals gives rise to the contents of GOT and GPT in serum in several tens of hour after administration. Kawaguchi, *et al.*[3] have shown in rats that those enzymes decreased in liver in contrast to a large increase in serum from 24 hr through 120 hr after intramuscular administration of CCl_4. It has been noted that the production of GPT of HTC cells is so much sensitive to CCl_4 treatment that both accumulation within cells and excretion into medium of that enzyme are decreased extensively, the former impaired much more. In conclusion it has been proved here that CCl_4 is capable of modifying the permeability of cell membrane to cellular transaminases directly at a surrounding concentration of 0.2 mM, altering also the rate of synthesis of the enzymes by cells more or less depending on the enzymes.

The HTC cell was derived from a hepatoma of the rat, handled through implantation in the rat ascites, but is found to maintain some histological and biochemical characteristics inborn in liver cell such as glucocorticoid-induced synthesis of tyrosine aminotransferase.[14] And now two pairs of isozymes of transaminase of that cell have proved of the same properties as those of the rat liver as far as separation profiles by DEAE cellulose column chromatography are concerned. Moreover simulation in a hepatic cell line in culture of the *in vivo* effect of CCl_4 on enzyme distribution in liver is very interesting in view of application of tissue culture of homogeneous mammalian cells to biochemical research as a model of biological system in cellular level. True applicability of the HTC cell as a model in vitro of the hepatic cell in toxicology will be examined by additional experiments elucidating the specificity of that effect of CCl_4 on the HTC cell as to cell types and enzymes in the cells as well as responses of the HTC cell to other hepato-toxic agents.

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