

EXPERIMENTAL STUDY ON ROLE OF LYSOSOMES IN CANCER CHEMOTHERAPY

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ABSTRACT

The lysosome has attracted much attention because of its role in the autolysis of cells, and has suggested the importance of introducing the concept of the lysosome into cancer chemotherapy.

In the present study acid deoxyribonuclease, β -glucuronidase and lysozyme in Yoshida ascites tumor cells were proved to be lysosomal enzymes because of the high latency in their activities and their characteristic intracellular distribution patterns. However, acid phosphatase was not characteristic in these respects, so that it is not reasonable to estimate the behaviors of lysosomes in Yoshida ascites tumor cells only by the activity of this enzyme.

Since the membranes bounding lysosomes have been assumed to be lipoprotein in nature, it was investigated whether lipoprotein lipase (LPL) could labilize lysosomes in tumor cells and it was found that LPL could be a strong lysosome labilizer *in vitro* and *in vivo*. A combined treatment with LPL and mitomycin-C (MMC) brought more remarkable changes in lysosomes of tumor cells than MMC alone. The membrane stability of lysosomes in tumor cells against LPL was less than that in liver cells of tumor-bearing rats, suggesting no enhancement of toxicity in the combination therapy with LPL and antitumor agents. The survival experiment showed that the cytotoxic effect of MMC was enhanced by LPL.

Based on these experimental findings clinical trials of combination chemotherapy with antitumor agents and lysosome labilizers are under investigation.

INTRODUCTION

As a result of biochemical studies of cell fractionation the concept of the lysosome was postulated in 1955 by de Duve and his co-workers.¹⁾ They called attention to a group of enzymes associated with particles of sedimentation properties distinct from microsomes or mitochondria and designated them "lysosomes". The enzymes of this group were all acid hydrolases and were practically unreactive to their respective substrates in intact preparations, and were released from granules subjected to graded activation by such as lysosome labilizers.

Lysosomes were shown to play a significant role in intracellular catabolic processes such as intracellular digestion, and physiological or pathological autolysis.²⁾

Several studies³⁻¹⁰⁾ revealed an elevation in lysosomal enzyme activities in tumor cells after treatment either with antitumor agents or irradiation, indicating that the lysosomes could play an important role in self-destruction of tumor cells in cancer therapy.

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Received for Publication June 30, 1976

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On the other hand, in clinical cancer chemotherapy it was shown that degeneration and autolysis of tumor cells were seen in the surgical materials when antitumor agents had been administered through bronchial or hepatic arteries in patients with primary lung cancer¹¹⁾ or metastatic cancer of liver¹²⁾, and that the changes of tumor cells were more remarkable in patients who had more favorable clinical responses¹³⁾. It was also recognized that some changes occurred in lysosomes of tumor cells within one hour after injection of mitomycin-C (MMC) to the tumor-bearing rats and the lysosomal enzymes of tumor cells seemed to be converted from the bound form to the free one⁴⁾. These results suggested the possibility that cytotoxic effects of antitumor agents might be enhanced by lysosome stabilizers, some lysosome stabilizers have already been shown to enhance the cytotoxic effects of antitumor agents.^{5), 6), 14-18)}

In the present study it was examined whether there could exist in Yoshida ascites tumor cells such particles which satisfied the concept of lysosome through the investigation of intracellular distribution patterns of acid deoxyribonuclease, acid phosphatase, β -glucuronidase and lysozyme.

Since the membranes bounding lysosomes have been assumed to be lipoprotein in nature,¹⁹⁾ it was examined whether lipoprotein lipase (LPL) could stabilize lysosomes in tumor cells *in vitro* and *in vivo*. A comparative study on the lysosome membrane stability of tumor cells and of liver cells of tumor-bearing rats against LPL was performed to examine the possibility of the enhancement of toxicity when lysosome stabilizers were combined with antitumor agents.

A survival experiment was also performed to evaluate the enhancement of cytotoxic effect of MMC by LPL.

MATERIALS AND METHODS

1. *Quantitative fractionation for definition of lysosome particles in Yoshida ascites tumor cells*

1) Fractionation

About 5×10^6 Yoshida ascites tumor cells were inoculated into the peritoneal cavities of male Donryu rats weighing 100 – 120 g. Four days after inoculation the rats were killed by a blow on the head. After opening the abdomen, the ascites was washed out by ice-cold physiological saline containing 1% disodium ethylenediaminetetraacetate (EDTA- Na_2), and the cells were collected by centrifugation at 1,500 r.p.m. for five minutes and washed a few times with ice-cold physiological saline. The tumor cells were suspended in an adequate amount of ice-cold physiological saline to calculate the cell count. The collected tumor cells were resuspended in ice-cold medium consisting of 0.25 M sucrose and 0.001 M EDTA- Na_2 to make 5×10^7 cells per ml. The tumor cells were twice homogenized with Emanuel-Chaikoff's homogenizer of 10μ clearance. The homogenate was centrifuged at 6,000 g-min to obtain sediment i.e., a nuclear fraction (N). The supernatant was a cytoplasmic extract (E), and an aliquot was used for analysis of enzyme activities. The remaining supernatant was further fractionated using a Kubota KR-66 preparative centrifuge. A heavy mitochondrial fraction (HM) was first sedimented from the cytoplasmic extract by centrifuging at 70,000 g-min. Then the supernatant was centrifuged at 260,000 g-min to separate a light mitochondrial fraction (LM). A microsomal fraction (P) was sedimented from the resulting supernatant by centrifuging at 3,000,000 g-min, using a Hitachi 55-PA preparative ultracentrifuge leaving the final supernatant (S).

Each fraction was used as a crude enzyme for the assay of acid deoxyribonuclease, β -glucuronidase, acid phosphatase, lysozyme, succinate dehydrogenase and adenosine triphosphatase activities. The sum of the values obtained separately from the nuclear fraction and the cytoplasmic extract was taken as representative of whole cells.

2) Expression of enzyme activities

The activities of the lysosomal enzymes were expressed as follows a) Free activities – These are the activities released from the lysosomal membranes and determined without blenderization or without adding Triton X-100. b) Total activities – These are the activities measured after quantitative release is obtained by adding Triton X-100 (Rohm & Haas Co., Philadelphia) at the concentration of 0.1 % (v/v). They are the sums of enzyme activities which are released from lysosomal membranes and contained in lysosomal particles. c) Bound (latent) activities – These represent estimates of the amount of intact particles present. They are the activities obtained by subtracting the free from the total activities. d) Unsedimentable activities – These are the total activities in the supernatant obtained by centrifuging at 3,000,000 g-min. They are the activities released from the binding to lysosomal membranes and subsequently solubilized into the cytoplasm.

In this paper, the unsedimentable activity, total and free activities in the sedimentable fraction are expressed as S, P and F, respectively. The sum of total activities in the unsedimentable and sedimentable fractions is expressed as T which indicates total activity in the cytoplasmic extract. (F + S) represents the free activity in the cytoplasmic extract.

3) Enzyme assays

a) Acid deoxyribonuclease: The enzyme assay was made by measuring the activities in the acid soluble fraction. Purified calf thymus deoxyribonucleic acid (Sigma Co., Ltd.) was used as a substrate. One half ml of the crude enzyme solution was added to 0.5 ml of deoxyribonucleic acid solution, of concentration 2 mg/ml, and 1 ml of 0.05 M citrate buffer solution (pH 5.2) containing 0.375 M sucrose was added to the above mixture. The reaction mixture was incubated for 15 minutes at 37°C and enzymatic reaction was stopped by adding 1 ml of ice-cold 2 N perchloric acid. The mixture was filtrated after standing for ten minutes in the ice-water bath. The activity of the enzyme was determined by measuring the amount of total phosphate in the filtrate by the method of Fiske-Subbarow²⁰. In the experiments 2 and 3, the extinction of the filtrate was read at 260 m μ and subsequently the amount of phosphate was calculated.

b) Acid phosphatase: The activity of this enzyme was measured by a modified method of Berthet *et al.*²¹ Three ml of the reaction mixture consisting of 1 ml each of 0.15 M β -glycerophosphate, 0.15 M acetate buffer solution (pH 5.0), and the crude enzyme solution was incubated for 15 minutes at 37°C. The reaction was stopped by adding 2 ml of ice-cold 10 % (w/v) trichloroacetic acid and the mixture was filtrated ten minutes later. The activity was determined by measuring the amount of inorganic phosphate in the filtrate by the method of Fiske-Subbarow²⁰. In the experiments 2 and 3, 0.02 M disodium para-nitrophenyl phosphate was used as a substrate instead of β -glycerophosphate. One half ml of the substrate solution was mixed with 0.25 ml of the crude enzyme solution and 1 ml of 0.1 M acetate buffer solution (pH 5.0). The enzymatic reaction was conducted in the same manner. The activity was determined by measuring the amount of para-nitrophenol, the product of the reaction. One ml of 1 M NaOH was added to 2 ml of the filtrate and then the solution was diluted with 7 ml of water. The extinction of the

diluted solution was read at 400 $m\mu$ and subsequently the amount of para-nitrophenol was calculated.

c) β -glucuronidase: Para-nitrophenyl glucuronide was used as a substrate²²⁾. Two ml of the reaction mixture consisting of 0.5 ml of 0.01 M para-nitrophenyl glucuronide, 0.5 ml of the crude enzyme solution and 1.0 ml of 0.1 M acetate buffer solution (pH 5.0) containing 0.375 M sucrose was incubated for 15 minutes at 37°C. The reaction was stopped by adding 2.0 ml of ice-cold 10%(w/v) trichloroacetic acid and the mixture was filtrated after standing for ten minutes in the ice-water bath. The activity of the enzyme was determined by measuring the amount of para-nitrophenol, the product of the reaction. One ml of 1 N NaOH was added to 2 ml of the filtrate and subsequently the extinction of the solution was read at 400 $m\mu$.

d) Lysozyme: Lysozyme activity was determined by modification of the method of Smolelis and Hartsell²³⁾. A suspension of the dried cells of *Micrococcus lysodeikticus* was prepared in 1/15 M phosphate buffer (Sørensen) of pH 6.2 such that, on a Leity-photometer (wave length of 640 $m\mu$), the percentage transmittance became 10%. Each 3 ml of the suspension of the enzyme was kept at 35°C for three minutes respectively, and then these were mixed. The mixture was kept at 35°C for ten minutes. The turbidity was read at 640 $m\mu$ and compared with the control which contained 1/15 M phosphate buffer instead of the enzyme. The decrease of the turbidity was expressed in terms of extinction.

e) Succinate dehydrogenase: The activity of this enzyme was determined by a modified method of Singer-Kearney²⁴⁾. Three ml of the reaction mixture consisting of 1.0 ml of 0.3 M phosphate buffer solution (pH 7.6), 0.2 ml of the crude enzyme solution, 0.3 ml of 0.01 M KCN, 0.1 ml of 1.0% phenazine metasulfate, 0.3 ml of 4×10^{-4} M 2, 6-dichlorophenol indophenol sodium, 0.2 ml of 0.3 M sodium succinate and 0.9 ml of water was incubated at 30°C. Decrease of unreduced 2, 6-dichlorophenol indophenol in the reaction mixture was measured by colorimetry. Then, the activity was estimated by calculating the amount of reduced 2, 6-dichlorophenol indophenol.

f) Adenosine triphosphatase (ATP ase): The activity was measured by a modified method of Skou²⁵⁾. The reaction mixture consisting of 0.2 ml of the crude enzyme solution and 1 ml of the substrate mixture (adenosine triphosphate 3×10^{-3} M, $MgCl_2$ 6×10^{-3} M, NaCl 100×10^{-3} M, KCl 20×10^{-3} M, EDTA- Na_2 1×10^{-3} M, histidine 30×10^{-3} M and sucrose 250×10^{-3} M, pH 7.6) was incubated for ten minutes at 37°C. The reaction was stopped by adding 0.2 ml of ice-cold 50%(w/v) trichloroacetic acid and the mixture was centrifuged at 2,500 r.p.m. for five minutes. The activity was determined by measuring the amount of inorganic phosphate in the supernatant by the method of Fiske-Subbarow²⁰⁾.

Enzyme activities were calculated per 1×10^7 cells or per mg total nitrogen. Total nitrogen contents of the enzyme solution were measured by using Nessler's reagent²⁶⁾.

2. Labilization of lysosomes by lipoprotein lipase (LPL)

1) Labilizing effect of LPL on lysosomes in tumor cells

The light mitochondrial fraction was separated as described above. An adequate amount of the light mitochondrial fraction corresponding to 4×10^8 cells/ml was suspended in 2 ml of the medium containing LPL, 0.25 M sucrose and 25 mM Tris-HCl buffer solution (pH 7.5), and incubated for 30 minutes at 30°C. The control was treated in an identical manner without addition of LPL to the incubation mixture. After incubation, it was diluted with 0.25 M sucrose containing 0.001 M EDTA- Na_2 to get a concentration of

2.5×10^7 cells/ml. The range of final LPL concentration was $0 \mu\text{g/ml}$ to $150 \mu\text{g/ml}$ and that of pH was 7.0 to 9.0.

An aliquot of the diluted suspension was assayed for released activities of acid deoxyribonuclease and β -glucuronidase which had been proved to be lysosomal enzymes in Yoshida ascites tumor cells, and the total activities of the two enzymes were also measured. The released activities were expressed as percentages of the total activities of the controls. The remainder of the diluted suspension was centrifuged at $3,000,000 \text{ g-min}$ and the supernatant was similarly assayed to determine the unsedimentable activities of both enzymes. The ratios of unsedimentable activities against released activities were calculated.

Enzyme activities were expressed as a value per 1×10^7 cells.

The activity of LPL was measured after incubating at 60°C for ten minutes to examine the stability of LPL against heat.

The LPL used here was extracted from *Mucor javanicus* as bile-sensitive lipase. The purity of this substance was determined by electrophoresis.²⁷⁾ It did not have any trypsin activity and was found to be not contaminated with phospholipase.²⁸⁾

2) Differences of lysosomal membrane stability between lysosomes in Yoshida ascites tumor cells and those in liver cells of tumor-bearing rats against LPL

Collection of Yoshida ascites tumor cells and separation of lysosomes were conducted according to the methods described above. Collection of liver cells and separation of lysosomes were conducted as follows: The livers having no metastases were extracted four days after inoculation of 5×10^6 Yoshida ascites tumor cells. The blood was drawn out and the livers were cut into small pieces, 10 g of which was suspended in 100 ml of ice-cold physiological saline solution containing 10% EDTA- Na_2 to make a 10% homogenate (w/v) with Potter's homogenizer. After centrifuging the homogenate at $8,600 \text{ g-min}$, the supernatant was recentrifuged at $240,000 \text{ g-min}$ to obtain a light mitochondrial fraction. The light mitochondrial fraction was suspended in a medium consisting of 0.25 M sucrose and 0.001 M EDTA- Na_2 so as to obtain 0.2 g of the wet liver in 1 ml. The suspension was used as the liver cell lysosome preparation.

The following procedure was conducted to compare the membrane stability against LPL between lysosomes in tumor cells and those in liver cells.

β -glucuronidase was chosen as a marker enzyme of lysosome.

LPL was dissolved in the medium containing 0.25 M sucrose and 25 mM Tris-HCl buffer solution, pH 7.5, varying in concentration from $5 \mu\text{g/ml}$, $12.5 \mu\text{g/ml}$, $25 \mu\text{g/ml}$, $50 \mu\text{g/ml}$, $100 \mu\text{g/ml}$ to $200 \mu\text{g/ml}$, respectively. One ml of each of the LPL solution in the same concentration was added to 1 ml of the lysosome preparation from tumor cells and to 1 ml of that from liver cells. After incubating for 30 minutes at 30°C , free activities of β -glucuronidase released in the medium through labilization by LPL were measured. Free activities of β -glucuronidase in the controls were measured in an identical manner without addition of LPL to the incubation mixtures in both lysosomal preparations. Total activities of both lysosome preparations in the controls were measured after treatment of the suspension with 0.1% Triton X-100 for ten minutes. Differences between the total and free activities (total activities – free activities) of each in the controls were estimated for bound activities of β -glucuronidase of lysosome preparations in the controls. From the above results the percentages of a series of β -glucuronidase free activities through labilization by LPL in various concentrations to bound activities of the controls were calculated

for the lysosomal membrane fragility (reciprocals of stability) against LPL.

3. *Changes of lysosomal enzyme activities in tumor cells following treatment with lipoprotein lipase (LPL) and/or mitomycin-C (MMC)*

About 5×10^6 Yoshida ascites tumor cells were inoculated into the peritoneal cavity of male Donryu rats weighing 100 – 120 g. Four days after inoculation the animals were used for the experiment. They were divided into the following four groups, i.e., groups of control, LPL alone, MMC alone and MMC combined with LPL. The control and LPL alone groups received a single intraperitoneal injection of 1 ml of distilled water and 5 mg/kg of LPL diluted with distilled water, respectively. One hour after injection, tumor cells were collected as described above. The MMC alone group received subcutaneously 5 mg/kg of MMC diluted with physiological saline and 30 minutes later 1 ml of distilled water intraperitoneally. The MMC combined with LPL group was treated with subcutaneous injection of MMC in a dose of 5 mg/kg of body weight and 30 minutes later treated with intraperitoneal injection of 5 mg/kg of LPL. After 30 minutes the tumor cells in each group were collected and homogenized as described above. The homogenate was centrifuged at 6,000 g-min to separate nuclei and cellular debris from a cytoplasmic component containing lysosome particles. The cytoplasmic component was further divided into two subfractions: sedimentable and unsedimentable fractions by centrifuging at 3,000,000 g-min using a Hitachi 55-PA preparative ultracentrifuge. These two fractions were diluted with the medium consisting of 0.25 M sucrose and 0.001 M EDTA- Na_2 , so that each ml contained cytoplasmic fractions from 1×10^7 cells. The methods of enzyme assay were as described above. Acid deoxyribonuclease, β -glucuronidase and acid phosphatase were chosen as lysosomal enzymes. Free and total activities of these enzymes in sedimentable fractions and total activities in unsedimentable fractions were measured as a value per 1×10^7 cells. In order to examine the influence of the treatment with LPL and/or MMC on lysosomal enzymes S/P ratios were calculated. In addition (F + S)/T ratios were also calculated. Activities of these hydrolases 15 hours after intraperitoneal injection of LPL in a dose of 5 mg/kg of body weight were also measured.

4. *Survival experiment*

In order to examine the influence of lipoprotein lipase (LPL) on the cytotoxic effect of mitomycin-C (MMC), a survival experiment was performed using male Donryu rats weighing 100 – 120 g. Yoshida ascites tumor cells were inoculated at a count of 2×10^7 cells into the peritoneal cavity of each rat. The animals were divided into the following groups, i.e., LPL alone, MMC alone, MMC combined with LPL and control groups. Forty-eight hours after inoculation, in the LPL alone group LPL in a dose of 5 mg/kg of body weight was administered into the peritoneal cavity. In the MMC alone group, MMC in a dose of 2 mg/kg was injected subcutaneously, and in the combination group, MMC in a dose of 2 mg/kg was injected subcutaneously and 30 minutes after the MMC injection LPL in a dose of 5 mg/kg was administered into the peritoneal cavity. The control group received an intraperitoneal injection of 1 ml of distilled water 48 hours after inoculation. Each group consisted of 20 rats. The animals were kept under observation for up to 30 days after inoculation of tumor cells.

Two mg of MMC per kilogram of body weight were employed in this experiment, because it had been recognized that a single subcutaneous injection of 2 mg/kg of MMC was the most effective dose for Yoshida ascites tumor bearing Donryu rats.¹⁴⁾

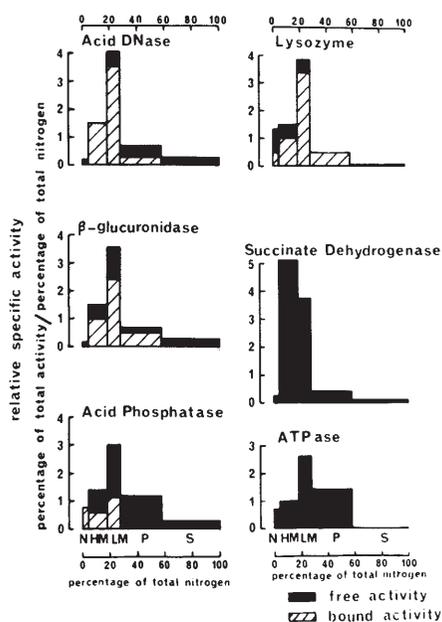


Fig. 1. Intracellular Distribution of Enzymes

RESULTS

1. Intracellular distribution patterns of enzymes in Yoshida ascites tumor cells

Fig. 1 illustrates the intracellular distribution patterns of the enzymes by plotting the relative specific activities in each fraction against their relative nitrogen content. The area of each block is proportional to the percentage of activity recovered in the corresponding fraction and its height to the degree of purification obtained for each fraction. Activities of acid deoxyribonuclease, β -glucuronidase and lysozyme were mainly distributed in the light mitochondrial fraction and their relative specific activities were highest in this fraction. In the cytoplasmic extract the latent activities of acid deoxyribonuclease and β -glucuronidase were found to be over 70% of their total activities and that of lysozyme to be about 60% of its total activity. On the contrary the intracellular distribution pattern of acid phosphatase was not so characteristic as the patterns of the other three acid hydrolases, and only less than 20% of the total activity was the latent activity of this enzyme (Fig. 2). However, the relative specific activity of this enzyme was highest in the light mitochondrial fraction and the bound activity was also highest in this fraction. The activity of succinate dehydrogenase was mainly distributed in both heavy and light mitochondrial fractions, but the relative specific activity of this enzyme was higher in the heavy mitochondrial fraction, so the intracellular distribution pattern was characteristic as that of a mitochondrial enzyme. ATPase was mainly distributed in the microsomal fraction, although the relative specific activity was highest in the light mitochondrial fraction. No bound activity of succinate dehydrogenase or ATPase was measured.

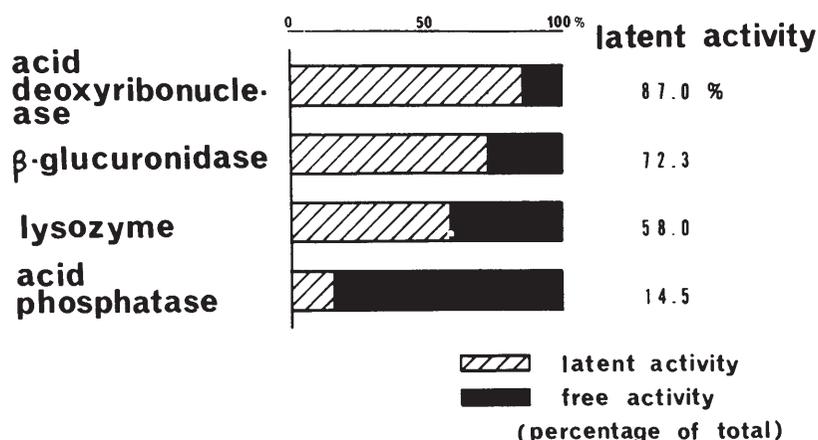


Fig. 2. Latent Activity of Acid Hydrolases in Yoshida Ascites Tumor Cells

2. Labilization of lysosomes by lipoprotein lipase (LPL)

1) Labilizing effect of LPL on lysosomes in tumor cells

Fig. 3 shows the free activities of acid deoxyribonuclease and β -glucuronidase released into the medium when the mixture of the lysosome preparation and LPL was incubated for 30 minutes at 30°C. The released activities are expressed as percentages of total activities of the controls. With increase in concentration of LPL, the released activities of both acid deoxyribonuclease and β -glucuronidase also increased and reached a plateau with the final concentration of over 125 μ g/ml of LPL. This finding indicates that lysosomes were labilized by LPL. Fig. 4 illustrates the influence of pH of the incubation medium on release of both enzymes by LPL. The release of the enzymes from lysosomes by

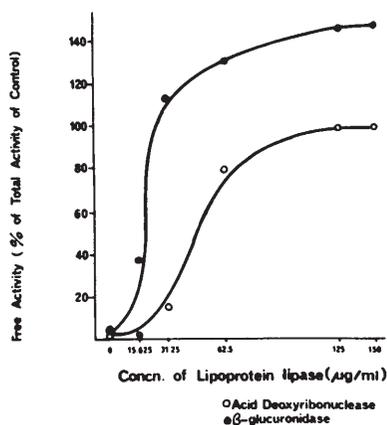


Fig. 3. Influence of Lipoprotein Lipase on Release of Lysosomal Hydrolases

Lysosomal preparation was incubated for 30 min at 30°C in 25 mM Tris-HCl buffer, pH 7.5 in the presence of 0.25 M sucrose and lipoprotein lipase.

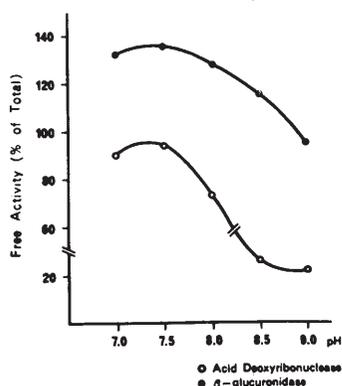


Fig. 4. Influence of pH of Incubation Medium on Release of Lysosomal Hydrolases by Lipoprotein Lipase

A light mitochondrial fraction was incubated for 30 min at 30°C in 25 mM Tris-HCl buffer in the presence of 0.25 M sucrose and lipoprotein lipase.

Table 1. Influence of Lipoprotein Lipase on Solubility of Lysosomal Hydrolases

Hydrolases	Activity released in the suspension (% of total activity of control)	Unsedimentable activity (% of released activity)	
		Control	Treated
Acid DNase	80.0	0	99.0
β -Glucuronidase	120.0	0	87.7

Lysosomal preparation was incubated for 30 min at 30°C in 25 mM Tris-HCl buffer (pH 7.5) in the presence of 0.25 M sucrose and 62.5 μ g/ml lipoprotein lipase. After removal of an aliquot of suspension for the measurement of enzyme activities, the suspension was centrifuged at 3,000,000 g-min and the supernatant was assayed similarly to determine the unsedimentable activities. The control was treated in an identical manner, without addition of lipoprotein lipase to the incubation mixture.

LPL was maximum at pH 7.5, the optimum pH of LPL itself. LPL heated at 60°C for ten minutes no longer had the activity to release the hydrolases from lysosomes. Therefore, it can be said that labilization of lysosomes by LPL is due to its enzymatic activity itself. Table 1 shows that almost 100 % of the free activity in acid deoxyribonuclease and more than 80 % in β -glucuronidase were recovered as unsedimentable activities in the supernatant, indicating that LPL not only labilized lysosomes but also solubilized hydrolases contained in lysosomes of Yoshida ascites tumor cells.

- 2) Differences of lysosomal membrane stability between Yoshida ascites tumor cells and liver cells of the tumor-bearing rats against LPL

Fig. 5 illustrates the labilizing effect of LPL on lysosomes in Yoshida ascites tumor cells and liver cells. About 10 % (percentage of the free against control bound activity) of lyso-

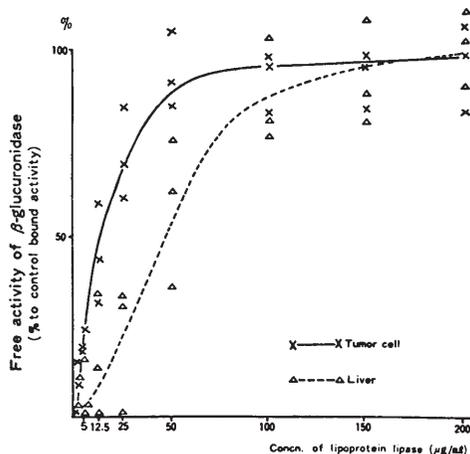


Fig. 5. Lysosomal Membrane Stability of Yoshida Ascites Tumor Cells and Liver Cells of Tumor-bearing Rats against Lipoprotein Lipase

Lysosomal preparation was incubated for 30 min at 30°C in 25 mM Tris-HCl buffer (pH 7.5) in the presence of 0.25 M sucrose and lipoprotein lipase. Control was treated in identical manner, without adding lipoprotein lipase to the incubation mixture.

somes in tumor cells was labilized at the concentration of 5 $\mu\text{g/ml}$ of LPL and about 50 % at 12.5 $\mu\text{g/ml}$, while only about 2 % of those in liver cells was labilized at 5 $\mu\text{g/ml}$ and 8 % at 12.5 $\mu\text{g/ml}$. Against lysosomes in tumor cells, LPL, with increase in concentration rapidly converts bound forms to free forms, but the conversion is gradual against lysosomes in liver cells. Comparing the lysosomal membrane stability of the two against LPL, it can be said that lysosomal membranes of Yoshida ascites tumor cells are more fragile than those of liver cells.

3. Changes of lysosomal enzyme activities in tumor cells following treatment with lipoprotein lipase (LPL) and/or mitomycin-C (MMC)

Unsedimentable total activities, and free and total activities in the sedimentable fraction of acid deoxyribonuclease, acid phosphatase and β -glucuronidase in the groups of LPL alone, MMC alone, MMC combined with LPL and control were measured after centrifuging at 3,000,000 g-min. S/P and (F + S)/T ratios are shown in Table 2 where activities of these acid hydrolases are expressed as a value per 1×10^7 cells. In the group of LPL alone, S/P and (F+S)/T ratios were more elevated than those in the control group in all except in the case of acid phosphatase one hour after injection. The elevation of S/P ratios was more remarkable than that of (F + S)/T ratios. In the group of MMC alone, S/P ratios were more elevated in all three hydrolases and (F + S)/T ratios were higher than those in the control group in all except in the case of β -glucuronidase. In each acid hydrolase the S/P ratio in the group of MMC combined with LPL was higher than in the group of LPL or MMC alone. This finding was most remarkable in acid deoxyribonuclease as was the case with (F + S)/T ratio. These results indicate that LPL has a property of labilizing lysosomes in tumor cells *in vivo* as well as *in vitro*, and also suggest that the labilization of lysosomes by MMC was enhanced by LPL. S/P and (F + S)/T ratios of the LPL alone group were not so different from those of the control group in all these enzymes 15 hours after intraperitoneal administration of 5 mg/kg of LPL. Therefore, the labilizing effect of LPL on lysosomes seemed to be transient.

Table 2. Influence of Mitomycin-C (MMC) and/or Lipoprotein Lipase (LPL) on the Release of Lysosomal Enzymes

	Control	Time after injection of MMC and/or LPL		
		LPL only 1	MMC only 1	MMC and LPL* 1
S/P				
DNase	0.04 \pm 0.01	0.10 \pm 0.02	0.08 \pm 0.01	0.28 \pm 0.03
β -glucuronidase	0.02 \pm 0.007	0.04 \pm 0.01	0.04 \pm 0.007	0.05 \pm 0.002
Acid Phosphatase	0.29 \pm 0.03	0.29 \pm 0.02	0.36 \pm 0.02	0.42 \pm 0.05
(F+S)/T				
DNase	0.33 \pm 0.02	0.45 \pm 0.10	0.47 \pm 0.10	0.52 \pm 0.06
β -glucuronidase	0.25 \pm 0.02	0.37 \pm 0.03	0.25 \pm 0.04	0.34 \pm 0.02
Acid Phosphatase	0.88 \pm 0.03	0.90 \pm 0.03	0.96 \pm 0.02	0.95 \pm 0.02

*LPL is injected 30 min. after MMC.

T: Total Activity in the Cytoplasmic Extract
 S: Total Activity in the Unsediment. Fraction
 P: Total Activity in the Sediment. Fraction
 F: Free Activity in the Sediment. Fraction

4. Survival experiment

As shown in Fig. 6, survival curves of the control and LPL groups were almost the same, the 50% survival time of the control group being 7.6 days and that of LPL group 7 days. The 50% survival time of the mitomycin-C (MMC) group was 15 days. The survival time of rats was further prolonged by the combination of LPL with MMC, the 50% survival time being 27 days. These results indicate that the cytotoxic effect of MMC was enhanced by LPL.

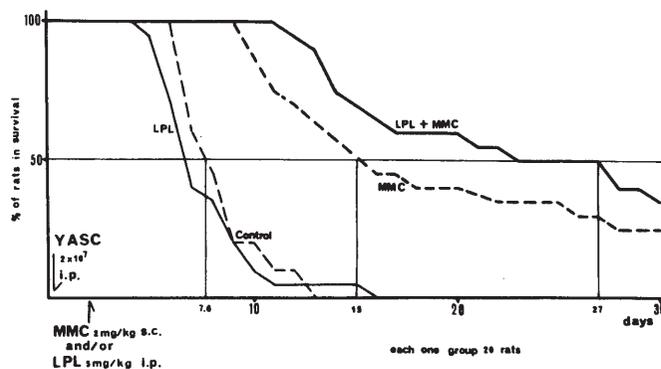


Fig. 6. Survival Rate of Yoshida Ascites Tumor bearing Rats Treated with Lipoprotein Lipase (LPL) and/or Mitomycin-C

DISCUSSION

Activities of acid deoxyribonuclease, β -glucuronidase and lysozyme in Yoshida ascites tumor cells mainly distributed in the light mitochondrial fraction and their relative specific activities were highest in this fraction. Latent activities of acid deoxyribonuclease and β -glucuronidase were found to be over 70% of their total activities and that of lysozyme to be about 60% of its total activity. On the contrary, the intracellular distribution pattern of acid phosphatase was not so characteristic as the patterns of the other three hydrolases, and only less than 20% of the total activity was the latent activity of this enzyme. The following biochemical characteristics are required for lysosomal enzymes. 1) Enzymes of this group are all hydrolases which have acid pH optima. 2) They share the property of "latency", i.e., they are practically unreactive toward their respective substrates in intact preparations, but become maximally active when they are treated with a variety of means. 3) These enzymes are localized in the light mitochondrial fraction and show different intracellular distribution patterns from those of microsomal or mitochondrial enzymes.¹⁾ Acid deoxyribonuclease, β -glucuronidase and lysozyme in Yoshida ascites tumor cells satisfy these characteristics, so they can be considered lysosomal enzymes. However, on acid phosphatase, because of its low latency and intracellular distribution pattern, it cannot be evaluated as a marker enzyme of lysosomes as far as Yoshida ascites tumor cells are concerned. Therefore, it may be unreasonable to estimate behaviors of lysosomes in Yoshida ascites tumor cells only by the activity of acid phosphatase.

It was recognized that the free and total activities of lysosomal enzymes increased following mitomycin-C (MMC) injection to the tumor-bearing rats.⁴⁾ It was also revealed

in the present study that released activities, i.e., S/P and (F + S)/T ratios, of acid deoxyribonuclease, β -glucuronidase and acid phosphatase increased one hour after MMC treatment. These results indicate that lysosomes play a role in the course of cellular degeneration and death of tumor cells through some mechanism. Sawant *et al.*²⁹⁾ reported that the enzymes located in lysosomes could work in the degradation and digestion of liver and its main components, mitochondria, microsomal fraction and nuclei. This finding suggests that lysosomes play a significant role in self-autolysis of cells. Therefore, the elevation of released activities of acid hydrolases one hour after MMC treatment is an important finding whichever mechanism, primary or secondary effects of MMC, prevails. These considerations suggest the possibility that some means such as lysosome labilizers which increase the released activities may further enhance the degeneration and degradation of tumor cells caused by MMC.

Niitani and his associates already tried to combine lysosome labilizers to enhance the antitumor effect of MMC. Plasmin was employed as a lysosome labilizer in experimental studies,¹⁴⁻¹⁷⁾ and urokinase,^{14),17)} which activated plasminogen to plasmin *in vivo*, was combined with MMC in clinical studies. The results of these studies indicated that the antitumor effect of MMC was enhanced by plasmin or urokinase. Brandes and his co-workers^{5),6)} treated transplantable carcinoma of mouse mammary gland with cyclophosphamide and vitamin A, a kind of lysosome labilizer, and showed that vitamin A enhanced the antitumor effect of cyclophosphamide. Niitani and his associates¹⁸⁾ also revealed that the antitumor effect of MMC was made greater by vitamin A.

In an effort to search for more effective lysosome labilizers, LPL was chosen in the present study, because the lysosomal membranes have been assumed to be lipoprotein in nature.¹⁹⁾ It was ascertained that LPL had a property of labilizing lysosomes in Yoshida ascites tumor cells *in vivo* as well as *in vitro*. In *in vivo* study it was proved by elevation of S/P ratios and (F + S)/T ratios following LPL treatment. This finding is not contradictory to the results of *in vitro* experiment where the majority of acid hydrolase activities released by LPL were found in the unsedimentable fraction. S/P ratios in the group of MMC combined with LPL were more elevated than those of MMC along group. The increases of S/P and (F + S)/T ratios caused by LPL were transient and these ratios were not so different from those of the control 15 hours after LPL treatment. The survival time of tumor-bearing rats following LPL treatment was almost the same as that of the control. These findings indicate that elevations of free activities, S/P or (F + S)/T ratios caused by LPL are not considered directly due to changes related to degeneration or death of tumor cells, and LPL has no cytotoxic effect. On the other hand the survival time of tumor-bearing rats following combined treatment with 5 mg/kg of LPL and 2 mg/kg of MMC was much longer than that treated with MMC in a dose of 2 mg/kg, the most effective dose for Yoshida ascites tumor bearing rats as a single dose.¹⁴⁾ These results indicate the possibility that the cytotoxic effect of MMC was enhanced through the acceleration of release of free-form lysosomal enzymes, which act in autolysis of cells, by concomitant administration of LPL. However, it was reported that hyperlipemia enhanced metastases of malignant tumor,³⁰⁾ and therefore, the antihyperlipemic action of LPL might inhibit metastases of tumor cells.

Thornes and his associates³¹⁾ demonstrated that the proteolytic enzyme brinase produced autocytotoxicity in patients with acute leukemia and its possible role in immunotherapy. Several lysosome labilizers were found to have adjuvant effects, increasing the antibody response in mice immunized with bovine serum albumin.³²⁾ Vitamin A alcohol was shown

to enhance the antitumor effect of 1, 3-bis(2-chloroethyl)-1-nitrosourea (BCNU) in murine L1210 leukemia. Direct observation of leukemic cells and ancillary experiments on red blood cells suggested that enhancement was secondary to a vitamin A - BCNU interaction within lipoprotein membranes of tumor cells, leading to irreversible and lethal membrane alteration.³³⁾ Studies have been made to inhibit proliferation and metastases of tumor cells by employing fibrinolytic activity of plasmin,³⁴⁻³⁶⁾ and a trial of plasmin treatment was done to increase intracellular concentration of an antitumor agent.³⁷⁾ Therefore, it cannot be concluded that the enhancement of cytotoxic effect of antitumor agents by lysosome stabilizers shown by the survival experiment is only due to stabilization of lysosomes. Anyway, it is very interesting in the clinical field that several agents which have been considered lysosome stabilizers have some other favorable properties in the treatment of cancer.

On the other hand it was reported that the influence of antitumor agents on lysosomes in tumor cells was greater than on those of normal liver cells.⁷⁾ Other investigators revealed that activities of lysosomal enzymes in tumor cells were higher than in normal cells.³⁸⁾ In the present study it has been shown that lysosomal membrane stability of tumor cells against LPL is less than that of liver cells of tumor-bearing rats. These facts may suggest that toxicity will not be enhanced by administration of lysosome stabilizers in clinical cancer chemotherapy which aims at killing of tumor cells.

A clinical trial of the combination chemotherapy with MMC and dextran sulfate,³⁹⁾ which could increase serum LPL level *in vivo*,^{40),41)} was performed to enhance the cytotoxic effect of MMC. Based on the results of combination therapies with MMC and urokinase,^{14),17)} and MMC and dextran sulfate, a combination chemotherapy with MMC, urokinase and dextran sulfate is under investigation. A preliminary report revealed a promising result.⁴²⁾

For the development of cancer chemotherapy, it is crucial to find new effective antitumor agents, but in practice it is also important to obtain better results by making the most use of antitumor agents currently available. Combination chemotherapy with various antitumor agents is one of the ways which aim to have additive or synergistic effects and less toxicity. The other way is combination of non-antitumor agents with antitumor agents to enhance the cytotoxic effects of the latter. Considerable numbers of studies have been performed to obtain more effects with this kind of combination therapy.^{5,6,14-18,33,37,43-49)} It is worth to consider the administration of lysosome stabilizers in cancer chemotherapy to enhance death of tumor cells as one of the combination therapies with non-antitumor agents and antitumor agents.

SUMMARY

Acid deoxyribonuclease, β -glucuronidase and lysozyme in Yoshida ascites tumor cells were proved to be lysosomal enzymes from the high latency in their activities and their characteristic intracellular distribution patterns. However, acid phosphatase in Yoshida ascites tumor cells was not characteristic in these respects. Therefore, differing from the case in liver or kidney cells, it is not reasonable to estimate the behaviors of lysosomes in Yoshida ascites tumor cells only by the activity of this enzyme.

It has been found that lipoprotein lipase (LPL) could stabilize lysosomes in tumor cells both *in vitro* and *in vivo*. A combined treatment with LPL and mitomycin-C (MMC) brought more remarkable changes in lysosomes of tumor cells than treatment with MMC

alone.

The membrane stability of lysosomes in tumor cells against LPL was less than that in liver cells of tumor-bearing rats. This finding suggests that toxicity may not be enhanced when lysosome labilizers are introduced into clinical cancer chemotherapy.

The survival experiment revealed that the tumor-bearing rats treated with MMC in combination with LPL survived much longer than those treated with MMC alone.

Based on these experimental results, lysosome labilizers are now being used in combination with antitumor chemotherapeutic agents to enhance the cytotoxic effects of the latter.

ACKNOWLEDGEMENT

The author is greatly indebted to Dr. Itsuro Sobue, Professor of the First Department of Internal Medicine, Nagoya University School of Medicine, and Dr. Kiyoji Kimura, Vice-director of National Cancer Center Hospital, for their guidance throughout this study. The author also expresses grateful acknowledgement to Dr. Hisanobu Niitani, Chief of Clinical Laboratory and Internal Medicine, National Cancer Center Hospital, and Dr. Masanori Shimoyama, Chief of Hematology, National Cancer Center Hospital, for their constant interest and direct guidance in this investigation. The author wishes also to thank Dr. Mamoru Sugiura, Professor of Tokyo College of Pharmacy, for generous supply of lipoprotein lipase from *Mucor javanicus*.

This study was supported in part by Grant-in-Aid for Cancer Research from the Ministry of Health and Welfare.

REFERENCES

- 1) DE DUVE, C., PRESSMAN, B. C., GIANETTO, R., WATTIAUX, R. & APPELMANS, F.: Tissue fractionation studies, 6. Intracellular distribution patterns of enzymes in rat-liver tissue, *Biochem. J.*, **60**, 604, 1955.
- 2) DE DUVE, C.: Lysosomes, a new group of subcellular particles, In *Subcellular Particles*, Edited by T. Hayashi, Ronald Press, New York, 1959, p. 128.
- 3) NIITANI, H., SUZUKI, A., SHIMOYAMA, M. & KIMURA, K.: Effect of mitomycin-C injection on lysosomal enzymic activities of Yoshida ascites sarcoma, *Gann*, **55**, 447, 1964.
- 4) NIITANI, H., SUZUKI, A., SHIMOYAMA, M. & KIMURA, K.: The role of lysosomes in cancer chemotherapy, II. Effect of mitomycin-C injection on lysosomal enzymic activities of Yoshida ascites sarcoma cells, *Gann*, **57**, 193, 1966.
- 5) BRANDES, D., ANTON, E., SCHOFIELD, B. & BARNARD, S.: Role of lysosomal labilizers in treatment of mammary gland carcinomas with cyclophosphamide (NSC-26271) — Preliminary report, *Cancer Chemother. Rep.*, **50**, 47, 1966.
- 6) ANTON, E. & BRANDES, D.: Lysosomes in mice mammary tumors treated with cyclophosphamide, Distribution related to course of disease, *Cancer*, **21**, 483, 1968.
- 7) AOSHIMA, M., TSUKAGOSHI, S. & SAKURAI, Y.: Biochemical changes in tumor cells caused by antitumor agents, II. Effect of several antitumor agents *in vivo* and *in vitro* on β -glucuronidase and two other lysosomal enzymes of Yoshida sarcoma cells, *Gann*, **57**, 279, 1966.
- 8) AOSHIMA, M., TSUKAGOSHI, S. & SAKURAI, Y.: Biochemical changes in tumor cells caused by antitumor agents, III. Further studies on the effects of antitumor agent on NAD'ase and a few lysosomal enzymes of Yoshida sarcoma cells, *Gann*, **58**, 75, 1967.
- 9) OBOSHI, S., SHIMOSATO, Y., ITAKURA, K. & UMEGAKI, Y.: Pathological changes following radiotherapy of cancer, *Igakuno-Ayumi*, **61**, 618, 665, 725, 1967. (in Japanese)
- 10) SHIMOSATO, Y. & WATANABE, K.: Enzymorphological observation on irradiated tumor, with a particular reference to acid hydrolase activity, I. Light microscopic study, *Gann*, **58**, 541, 1967.
- 11) OGATA, Y., YONEYAMA, T., HASEGAWA, H., FUJII, K., SHIMOSATO, Y., YAMAMOTO, H.,

- NARUKE, T., SHIBATA, H. & SUEMASU, K.: Chemotherapy of lung cancer by the bronchial infusion, *Hai to Shin*, **13**, 316, 1966. (in Japanese)
- 12) ITO, I., HATTORI, T. & KOYAMA, Y.: Treatment of metastatic liver cancer, In *Cancer Chemotherapy*, Edited by S. Ishikawa, Ishiyaku Press, Tokyo, 1966, p.328, (in Japanese)
 - 13) SHIMOSATO, Y., BABA, K., OBOSHI, S., OGATA, T., YONEYAMA, T., SUEMASU, K. & ISHIKAWA, S.: Pathological studies on pulmonary carcinomas treated with mitomycin-C bronchial artery infusion, *Jap. J. Cancer Clin.*, **14**, 945, 1968. (in Japanese)
 - 14) TANIGUCHI, T.: Experimental and clinical studies on cancer chemotherapy — Enhancement of the cytotoxic effect of mitomycin-C by one of lysosome stabilizers, plasmin — *J. Jap. Cancer Therp.*, **7**, 32, 1972. (in Japanese)
 - 15) SHIMOYAMA, M., NIITANI, H., TANIGUCHI, T., INAGAKI, J. & KIMURA, K.: Lysosome and cancer chemotherapy, III. Synergic effect of mitomycin-C and one of lysosome stabilizers, plasmin, *Igaku-no-Ayumi*, **65**, 349, 1968. (in Japanese)
 - 16) SHIMOYAMA, M., NIITANI, H., TANIGUCHI, T., INAGAKI, J. & KIMURA, K.: The role of lysosomes in cancer chemotherapy, III. Influence of plasmin on the lysosomes of tumor cells and on the cytotoxic effect of mitomycin-C, *Gann*, **60**, 33, 1969.
 - 17) NIITANI, H., SUZUKI, A., KONDA, C., TANIGUCHI, T., INAGAKI, J. & KIMURA, K.: Synergic effects of lysosome stabilizer and anti-neoplastic agents with special reference to the effects of plasmin and mitomycin-C, *Saishin Igaku*, **23**, 2168, 1968. (in Japanese)
 - 18) NIITANI, H.: Effect of combined chemotherapy with lysosome stabilizers and antitumor agents. *J. Jap. Cancer Therp.*, **4**, Supplement, 35, 1969. (in Japanese)
 - 19) BEAUFAY, H.: Liberation of lysosomal enzymes by enzymatic agents, *Arch. Internat. Physiol. Biochim.*, **65**, 155, 1956.
 - 20) FISKE, C. H. & SUBBAROW, Y.: The colorimetric determination of phosphorus, *J. Biol. Chem.*, **66**, 375, 1925.
 - 21) BERTHET, T. & DE DUVE, C.: Tissue fractionation studies, I. The existence of a mitochondrial-linked enzymically inactive form of acid phosphatase in rat-liver tissue, *Biochem. J.*, **50**, 174, 1951.
 - 22) KATO, K., YOSHIDA, K., TSUKAMOTO, H., NOBUNAGA, M., MASUYA, T. & SAWADA, T.: Synthesis of p-Nitrophenyl β -D-Glucopyranosiduronic acid and its utilization as a substrate for the assay of β -glucuronidase activity, *Chem. Pharmac. Bull.*, **8**, 239, 1960.
 - 23) SMOLELIS, A. N. & HARTSELL, S. E.: The determination of lysozyme, *J. Bacteriol.*, **58**, 731, 1949.
 - 24) SINGER, T. P. & KEARNEY, E. B.: Determination of succinate dehydrogenase activity, In the *Methods Biochem. Anal.*, **4**, 307, 1957.
 - 25) SKOU, J. C.: The influence of some cations on an adenosine triphosphatase from peripheral nerves, *Biochim. Biophys. Acta*, **23**, 394, 1957.
 - 26) WICKS, L. F.: A cheaper Nessler's reagent by the use of mercuric oxide, *J. Lab. Clin. Med.*, **27**, 118, 1941.
 - 27) OGISO, T. & SUGIURA, M.: Studies on bile-sensitive lipase, V. Purification and properties of lipase from *Mucor javanicus*, *Chem. Pharm. Bull.*, **17**, 1025, 1969.
 - 28) SUGIURA, M.: Personal communication.
 - 29) SAWANT, P. L., DESAI, I. D. & TAPPEL, A. L.: Digestive capacity of purified lysosomes, *Biochim. Biophys. Acta*, **85**, 93, 1964.
 - 30) CLIFFTON, E. E., AGOSTINO, D. & MINDE, K.: The effect of hyperlipemia on pulmonary metastases of Walker 256 carcino-sarcoma in the rat, *Cancer Res.*, **21**, 1062, 1961.
 - 31) THORNES, R. D., DEASY, P. F., CARROLL, R., REEN, D. J. & MACDONELL, J. D.: The use of the proteolytic enzyme brinase to produce autotoxicity in patients with acute leukemia and its possible role in immunotherapy, *Cancer Res.*, **32**, 280, 1972.
 - 32) SPITZNAGEL, J. K. & ALLISON, A. G.: Mode of action of adjuvants, Retinol and other lysosome-stabilizing agents as adjuvants, *J. Immunol.*, **104**, 119, 1970.
 - 33) COHEN, M. H. & CARBONE, P. P.: Enhancement of the antitumor effects of 1,3-bis (2-chloroethyl)-1-nitrosourea and cyclophosphamide by vitamin A, *J. Nat. Cancer Inst.*, **48**, 921, 1972.
 - 34) CLIFFTON, E. E. & AGOSTINO, D.: Cancer cells in the blood in simulated colon cancer, resectable

- and unresectable, effect of fibrinolysin and heparin on growth potential, *Surgery*, **50**, 395, 1961.
- 35) CLIFFTON, E. E.: Effect of fibrinolysin on spread of cancer, *Fed. Proc.*, **25**, 89, 1966.
 - 36) LARSEN, V., MOGENSEN, B., AMRIS, C. J. & STORM, O.: Fibrinolytic enzyme in the treatment of patients with cancer, *Dan. Med. Bull.*, **11**, 137, 1964.
 - 37) KONDA, C.: Clinical and experimental studies on cancer chemotherapy with special reference to mitomycin-C and its combination chemotherapy, *J. Jap. Cancer Therp.*, **3**, 259, 1968. (in Japanese)
 - 38) DE DUVE, C.: Lysosomes and chemotherapy, In *Biological Approaches to Cancer Chemotherapy*, Edited by R. J. C. Harris, Academic Press, New York, 1961, p.101.
 - 39) NIITANI, H., INAGAKI, J., TANIGUCHI, T., SHIMOYAMA, M., KONDA, C., SUZUKI, A., TAKIGUCHI, M. & KIMURA, K.: Effect of combined therapy with lysosome labilizers and anti-neoplastic agents with reference to increased effect of mitomycin-C by lipoprotein lipase, *Saishin Igaku*, **25**, 172, 1970. (in Japanese)
 - 40) ROBINSON, D. S., HARRIS, P. A. & RICKETTS, C. R.: The production of lipolytic activity in rat plasma after the intravenous injection of dextran sulfate, *Biochem. J.*, **71**, 286, 1959.
 - 41) YAMADA, K. & KUZUYA, F.: Clinical trial of heparin and heparinoid, *Saishin Igaku*, **17**, 2605, 1962. (in Japanese)
 - 42) NIITANI, H., SUZUKI, A., TANIGUCHI, T., SAIJO, N., KAWASE, I. & KIMURA, K.: Effect of combination treatment with mitomycin-C and lysosome labilizers on nodular pulmonary metastases, *Gann*, **65**, 403, 1974.
 - 43) SHIBATA, K., SATO, S., EZAKI, R., FUKUHARA, T., TANAKA, A., HAYASHI, J., FUNAHASHI, K. & SATO, T.: Practical problems in cancer chemotherapy — Relationship between tissue concentration of antitumor agents and their effects, *Jap. J. Clin. Exp. Med.*, **47**, 639, 1970. (in Japanese)
 - 44) SUZUKI, M., YAMAURA, H., ABE, I. & SATO, H.: Experimental studies on local penetration of drugs in cancer chemotherapy, *Proc. Japan Cancer Asso.*, **28**, 233, 1969. (in Japanese)
 - 45) KIKUCHI, K., ANEHA, Y., NATSUME, R., KANNO, H. & KUNII, Y.: Clinical study for augmentation of efficacy of anticancer drugs (1st Report) — Combination therapy by proteolytic enzyme and anticancer drugs, *J. Jap. Cancer Therp.*, **7**, 270, 1972. (in Japanese)
 - 46) KIMURA, I., OTA, Z., KAGEYAMA, H., KAWANISHI, K., KODANI, H., MORI, T., MORITANI, Y., NAGAIRO, S., HAYASHI, N., YAMANA, M., ONOSHI, T. & NISHIZAKI, Y.: Studies on the mechanism of drug action of chloroquine on malignant tumors (II), *Proc. Japan Cancer Asso.*, **23**, 138, 1964. (in Japanese)
 - 47) TSUJI, S., IRIE, K., TOMINAGA, S., FUJITA, K., WATANABE, Y., KOMOGUCHI, H. & OTA, T.: Studies on the chemotherapy of malignant neoplasm, especially on the chemotherapy combined with chloroquine, *Proc. Japan Cancer Asso.*, **23**, 145, 1964. (in Japanese)
 - 48) RAUEN, H. M. & KRAMER, K. P.: Total blood concentration of alkylating agents in rats after administration of cyclophosphamide and acyclophosphamide, *Drug-Res.*, **14**, 1066, 1964.
 - 49) GAUDIN, D. & YIELDING, K. L.: Response of a "resistant" plasmacytoma to alkylating agents and X-ray in combination with the "excision" repair inhibitors caffeine and chloroquine, *Proc. Soc. Exp. Biol. Med.*, **131**, 1413, 1969.