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Cytotoxic Factor from Mouse Liver

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An extract from mouse livers showed cytotoxic activity against Ehrlich ascites tumor cells. The activity was associated neither with proteins nor nucleotides in the extract. The active substance was heat-stable and appeared as a lipid, being extractable with organic solvents. It was slowly adsorbed to the cells during incubation to exert the cytotoxicity. The adsorption was inhibited in the presence of glucose.

INTRODUCTION

The presence of cytotoxic substances has been found in normal and tumor tissues of animals. Some of them may relate to tumor resistance in host animals. They include proteinous lymphotoxins from sensitized lymphoid cells (Granger *et al.*, 1973; Peter *et al.*, 1973; Williams and Granger, 1968), unknown substances to cause cachexia in patients bearing cancer (DeWys, 1970) and hepatic chalones (Thornley and Lawrence, 1975; Verly *et al.*, 1971). A probable biogenetic amine from normal tissues has been known to suppress the growth of L cells by inhibiting protein synthesis (Borner, 1976). Another low molecular weight compound from liver has been reported to inhibit the DNA synthesis of hepatoma cells (Sekas and Cook, 1976). On the other hand, sub-cellular fractions from liver phagocytic cells of mice inoculated with BCG have been studied to find a fraction which causes the lysis of tumor-cells (Ferluga and Allison, 1975).

In previous papers, we have reported the presence and the mode of action of a proteinous cytotoxic substance in the microsomal fraction of mouse spleen (Momii, *et al.*, 1979 a, b). Finding further a cytotoxic activity in the extract of normal mouse liver, we report some properties of the cytotoxic factor in this paper. In contrast with the spleen factor, the liver factor appears as a lipid.

EXPERIMENTAL

Cytotoxicity test

The cytotoxicity was defined as injurious change in the permeability of cell membrane and was assayed by dyeing the Ehrlich ascites tumor cells (E-

cells) with nigrosine. E-cells harvested from ddN or ddd female mice were washed with a Ca^{2+} , Mg^{2+} -free phosphate-buffered saline solution (PBS(-)) or a 50 mM Tris-HCl buffer solution and were suspended in the same solution to adjust the cell density as 1.3×10^8 cells/ml. A test sample (0.5 ml) and the cell suspension (0.01 ml) were mixed in a 10-ml test tube and were incubated at 37°C with shaking. The test samples were always dissolved in and dialyzed, if necessary, against the same solution as that the cells were suspended. After 2 hr incubation 1.5 ml of saline containing 0.4% nigrosine was added to the mixture and cell number was counted on a hematometer. In order to correct possible errors owing to the autolysis of dead cells, the injured cell number (%) was calculated as follows:

$$\text{Injured cells (\%)} = 100(L_c - L_t \times T_c / T_t)L_c$$

where L and T denote the unstained (living) and total cell numbers, and the suffixes c and t mean control and test, respectively. In the control the test sample was replaced with PBS(-) or the buffer solution. The experiment was duplicated.

The specific activity was represented as the reciprocal of absorbance at 280 nm (SA_{280}), or of weight (mg) per ml (SA_w), of the test sample to injure 50% of the cells.

Preparation of mouse liver extracts

Livers from ddN female mice were sliced and homogenized in PBS(-) with a teflon homogenizer. To a 10, 000xg supernatant obtained from the homogenate was added a saturated ammonium sulfate solution (30 % saturation) and the resulting precipitate was dialyzed against PBS(-) by using a Visking tube to get a fraction, "Liver-30".

Liver-30 was treated for 30 min with a 59mM Tris-HCl buffer containing 500 mM KCl, 5 mM MgCl_2 , and 0.18 % sodium deoxycholate. The mixture was centrifuged at 105, 000xg for 60 min and the supernatant was submitted to a Sephadex G-25 column (2.6 x 51.7 cm) which had been equilibrated with PBS (-). The UV absorbing fractions eluted with PBS(-) were pooled and back-dialyzed against solid ammonium sulfate to make precipitation. The precipitate was dissolved in and dialyzed against PBS(-) for the cytotoxicity assay.

Lipid extraction

The microsome fraction of mouse liver homogenate was obtained by a usual manner at 100, 000xg. To the microsome suspension in PBS(-) was added saturated ammonium sulfate to make 30 % saturation. The precipitate, "Microsome-30", was dialyzed against PBS(-) for the assay.

The dialyzed microsome- was lyophilized and extracted with a chloroform-methanol (1 : 2) mixture at 4°C with shaking for 60 min according to Bligh-Dyer's method (Fujino, 1978). The insoluble materials were again extracted with a chloroform-methanol-water (1 : 2 : 0.8) mixture. The combined extracts were mixed with chloroform and water and the chloroform layer was separated.

Extraction of cytotoxic factor

Mouse livers (77.4 g) were homogenized in 154.8 ml of acetone in a homogenizer (Teraoka, type D) and filtered. The acetone was removed with a rotary evaporator under reduced pressure to get a residue (Acetone extract). The acetone extract was dissolved in benzene and fractionated with 2% tartaric acid and 2% sodium bicarbonate into basic, acid and neutral fractions according to a usual manner.

Time course of cytotoxic action

The reaction mixtures of Microsome- ($A_{280}=0.92$) and E-cells (1.3×10^6) in PBS(-) were incubated at 37°C for 30, 60 and 120 min, respectively. After the incubation, dead cells were counted.

Adsorption of the cytotoxic factor on E-cells

The concentrations of Microsome- in PBS(-) were adjusted to 1.12, 3.37 and 37.3 in absorbance at 280 nm. E-cells (3×10^7) killed by heating at 80°C were added to 1.5 ml of the Microsome- solutions. The mixtures were incubated at 37°C for 60 min and centrifuged. The supernatant was subjected to the cytotoxicity test.

Inhibition of cytotoxic action with glucose

The mixture of Microsome- ($A_{280}=1.12$) and n-glucose in PBS(-) was assayed for the cytotoxicity.

For the time course study of interaction among glucose, the cytotoxic factor and E-cells, 0.25 ml of 6.7 mM glucose was added to the mixture of Microsome-30 ($A_{280}=1.94$; 0.25 ml) and E-cell suspension (0.01 ml) which had been incubated at 37°C for an appropriate time. After incubation for 120 min in total, dead cells were counted.

RESULTS AND DISCUSSION

Cytotoxicity of liver fractions

Living cells were not dyed with nigrosine. Cells whose cell membrane was damaged by the action of a toxic substance incorporate the dye and the dyed cells then lyse. Thus, "dyed cell" is regarded as a synonym for "dead cell" in this paper.

The 10,000 $\times g$ supernatant of mouse liver extract with PBS(-) showed such a cytotoxic activity against Ehrlich ascites tumor cells; the specific activity based on A_{280} (SA_{280}) was 1.1. Ammonium sulfate precipitated the cytotoxic factor at 30% saturation, giving the fraction "Liver-30" ($SA_{280}=2.3$). The precipitate of Liver-30 at 105,000 $\times g$ (microsomal fraction) had an SA_{280} of 3.4 but the solubilized fraction obtained by treating it with deoxycholate was inactive.

On the other hand, the chloroform-methanol extract of mouse liver microsomes (Microsome-30), whose specific activity based on mg/ml (SA_w) was 2.1, showed a high cytotoxicity ($SA_w=17.5$), suggesting that the cytotoxic factor

is a kind of microsomal lipids.

Since preparing the microsomal fraction is unsuitable to obtain mass of the cytotoxic substance, we examined to extract it directly from the liver. Direct extraction with a chloroform-methanol mixture according to Wada and Sugano (1972) and Kates (1972) was unsuccessful, while the acetone was effective to extract the cytotoxic factor from livers.

The acetone extract was fractionated into acid, basic and neutral fractions. Only the neutral fraction had the cytotoxic activity (Table 1). Its specific activity ($SA_w=18.5$) is comparable to that of the chloroform-methanol extract of the microsomes. The total activity found in the neutral fraction was 9 times as much as that in the acetone extract, suggesting that some inhibitory substances may be removed by treating with acid and base.

Table 1. Fractionation of mouse liver cytotoxic factor.

Fraction	Yield (g)	Specific activity ¹	Total activity
Acetone extract	10.4	0.54	5.6
Neutral fraction	2.7	18.5	49.9

1) Reciprocal of mg/ml to kill 50 % of cells.

Properties of the mouse liver cytotoxic factor

Since the cytotoxic factor found from mouse spleen was sensitive to protease to lose the cytotoxicity (Momii *et al.*, 1979 a, b), we examined the effects of some enzymes against the present liver cytotoxic factor, using Liver-30. When it was incubated with a non-specific protease from *Streptomyces griseus* (pronase, Sigma Chemical Co.) or nuclease P₁ at 37°C for 60 min, no cytotoxic activity was lost (Table 2). Thus, neither protein nor nucleic acids may associate with the cytotoxicity of the liver factor.

The thermostability of Liver-30 was also tested. The specific activity increased a little by heat treatments; it did 1.1 and 1.56 times by heating at 60 and 100°C, respectively, for 30 min. When Liver-30 heated at 100°C was dialyzed against PBS(-) for 9 hr, the cytotoxic activity did not change owing probably to the insolubility of the factor in aqueous media. From these findings and the results of extraction with organic solvents, it was concluded that the liver-cytotoxic factor should be a lipid.

Table 2. Effects of heat and enzymes on cytotoxic activity.

Sample	Treatment with	Specific activity ¹
Liver-30	none	1.2
Liver-30	heating ²	1.5
Heated Liver-30	pronase	1.9
Liver-30	nuclease P ₁	1.9

1) Reciprocal of absorbance at 280nm to kill 50% of cells.

2) Heated at 100°C for 30 min, the sample was centrifuged to get the supernatant (heated Liver-30) for cytotoxicity assay.

This liver cytotoxic factor is apparently different from the proteinous cytotoxic factor of mouse spleen (Momii *et al.*, 1979 b) and also from hepatic chalon which is labile to proteinase (Thornley and Laurence, 1975). Barra *et al* (1979) found water-soluble cytotoxic factors from liver which consisted of thermo-labile biopolymer and stable factor. The latter looks similar to the present cytotoxic factor, but they differ each other in the point of water-solubility.

Ferluga and Allison (1975) have reported that plasma membrane from lymphoid cell systems has cytotoxic activity to cause the lysis of cells, provided the cell contacts with the membrane, indicating the importance of plasma membrane for immunological response. It is interesting in this context to note that the present cytotoxic factor active against E-cells was obtained from the microsomal fraction, a kind of plasma membranes, of normal liver cells.

Mode of action

A time course study in the cytotoxic action of Microsome- showed a lag time of about 30 min (Fig. 1). This may be explained if the slowly adsorbed factor triggers a prompt cell-damage as soon as exceeding a threshold of adsorption on the cell. The cytotoxic factor was actually adsorbed to E-cells, even after killed by heat. The amount of the factor (Microsome-30; A_{280} =ca. 1) corresponding to kill 100 % of E-cells assayed was completely lost by adsorption to 3×10^6 cells which had been killed by heat (Fig. 2).

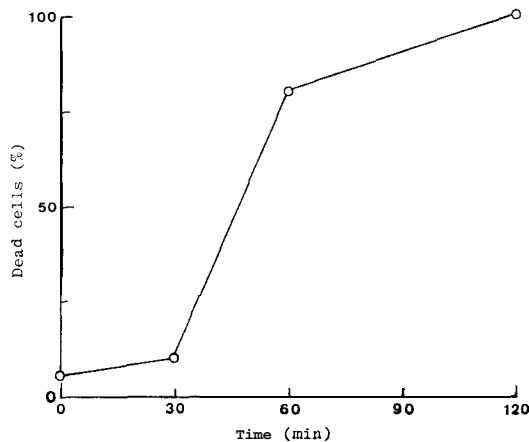


Fig. 1. Time course for the cytotoxic action of Microsome- at 37°C.

The cytotoxic activity of Microsome- was inhibited in the presence of glucose, provided that glucose and the cytotoxic factor were simultaneously mixed with E-cells (Fig. 3). The inhibition was complete at 3.3 mM of glucose for Microsome- whose absorbance at 280 nm was 1.12. The cytotoxicity of Microsome- at this concentration was 100 % in the absence of glucose.

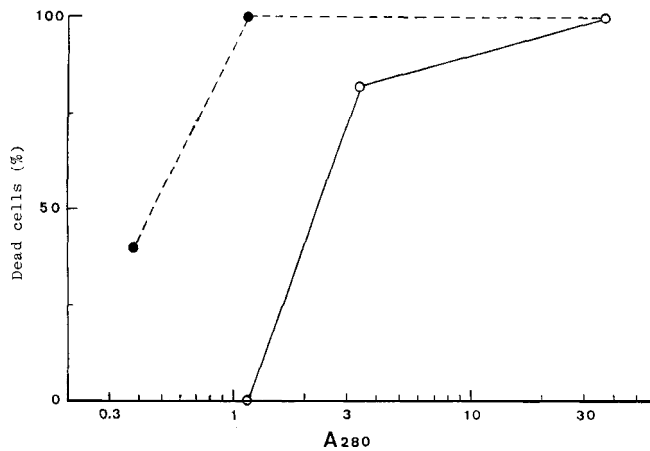


Fig. 2. Adsorption of cytotoxic factor to E-cells.

○—○: After Microsome- was incubated with E-cells killed by heating for 60 min, the cells were spun down and the supernatant was assayed for cytotoxicity. A_{280} indicates the values before treating for the adsorption.
 ●- - -●: Control.

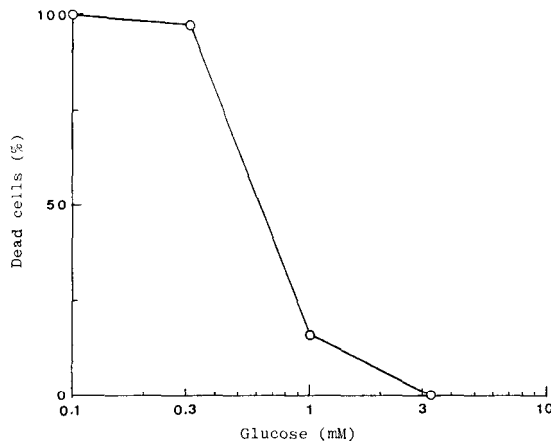


Fig. 3. Inhibition of cytotoxic activity with glucose.

The concentration of Microsome- used ($A_{280}=1.12$) was enough to kill 100% of E-cells in the absence of glucose.

When E-cells had been preincubated with the factor before adding glucose (3.3 mM), the inhibitory activity of glucose was seen by 30min preincubation, whereas any effect of glucose was not observed in preincubation longer than 60 min (Fig. 4).

Tumor cells are known to have a great affinity for glucose (Shapot, 1972). It may inhibit the adsorption of the cytotoxic factor to the cells but can not rescue the cell-damage affected by the adsorbed cytotoxic factor.

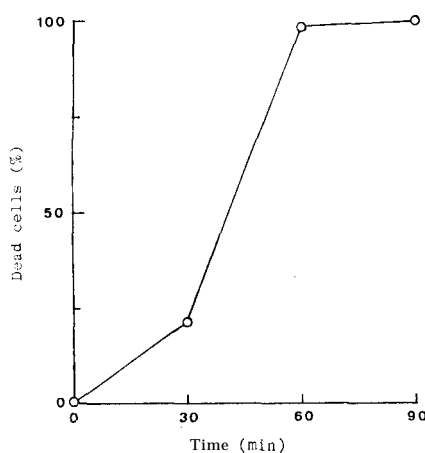


Fig. 4. Time course for the inhibitory action of glucose on the cytotoxic activity of Microsome-30.

The abscissa indicates preincubation time before addition of glucose (3.3 mM). The ordinate indicates the cytotoxic activity of Microsome- ($A_{280} = 0.98$).

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