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Mode of Action of a Cytotoxic Factor in Mouse Spleen

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The processes by which Ehrlich ascites tumor cells are killed were examined, and the mode of action of the cytotoxic factor was discussed. As a result, it was inferred that the cytotoxic factor was adsorbed on the surface of the target cells within 15min. and then transferred into the intracellular space through endocytosis mediated by a colchicine-sensitive structure. or microtubules. The cytotoxic factor which entered into the cytoplasm is expected to inhibit a metabolism in the target cells.

Various substances affect the emergence of cell injuring activity of some cytotoxic or cytolytic substances. For example, cytolysis by complement-fixation reaction requires divalent cations (Inoue, 1973) and agglutination by some lectins also requires them (Wallach, 1975).

On the other hand, cytotoxicity and agglutination by lectins are inhibited by some sugars (Refsnes et *al.,* 1974; Nicolson et *al.,* 1975; Kawaguchi, 1976). This may be due to competitive inhibition of adsorption of lectin molecules on the surface of the target cells.

Inhibitory drugs of the microtubular system, such as colchicine and ***vinca*** alkaloids, also depress the agglutinating action of lectins (Berlin and Ukena, 1972; Yin *et al., 1972).* Additionally endocytosis of lectins is necessary for the cytotoxic action (Refsnes *et al., 1974;* Nicolson *et al., 1975).* The drugs would be responsible for the inhibition of clustering of their receptors and endocytosis of lectin-receptor complex into the cells (Berlin, 1975).

The actions of lectins on cells, such as agglutination and cytotoxicity, are also depressed at low temperature (Nicolson, 1972; Noonan and Burger, 1973; Olsnes ***et al., 1973).***

Cell injuring reaction caused by killer T-cells is also affected by external factors described above (Hashimoto, 1975).

Cytotoxicity of the microsomal fraction obtained from mouse spleen extract was reported previously (Momii *et al.,* 1979). In this report the pro-

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cesses by which target cells are killed were examined, and the mode of action of the cytotoxic factor was discussed.

MATERIALS AND METHODS

Preparation of mouse spleen extract

Mouse spleen extract was prepared as described in the preceding paper (Momii *et al., 1979).* A detailed fractionation and the description of fractions (R-l-a, RP-30, NP-30 etc.) were also shown in that paper.

Cytotoxicity test

E-cells harvested from the peritoneal cavity of a mouse which had been inoculated the cells were washed with PBS(-) or VBS (veronal buffered saline) several times and the concentration was adjusted to 8x 10^7 cells per ml. One hundredth ml of the suspension and 0.5 ml of the active fraction of the spleen extract were mixed and incubated at 37°C for 2 hours with successive shaking in duplicate test tubes. The dead cells were stained with 1. 5 ml of 0.4 % nigrosin solution, and estimated with a Bürkel-Türk hematometer. A time-course study was carried out with minimal concentration of RP-30 preparation that could give 100 % of the dead cells in 2 hours of incubation.

Effect of temperature and shaking on the cytotoxicity

The experimental conditions were as follows, namely at 0°C under successive shaking, and at 37°C or 0°C under occasional shaking at 20 minutes-intervals.

Effect of divalent cations on the cytotoxicity

The cytotoxicity with and without divalent cations was tested. The cytotoxicity in the presence of the cations was assayed as follows. RP-30 preparation was diluted with VBS to have the absorbance at 280 nm from 1.0 to 0.2 (about 30 to 100 times dilution, respectively). One hundredth ml of the suspension of E-cells provided with VBS was mixed with the RP-30 preparation, and incubated at 37°C for 2 hours. Concentration of Ca^{2+} and Mg^{2+} in VBS are 0.25 and 0.83 mM, respectively. The cytotoxicity in the absence of the cations was assayed with the cell suspension provided with PBS(-) containing 10 mM of EDTA, and RP-30 preparation diluted with the same saline.

Adsorption of the cytotoxic factor on the target cells

The adsorption test of the cytotoxic factor on E-cells was carried out at 37°C or at 0°C. One tenth ml of E-cell suspension containing 8×10" cells were centrifuged at 10,000 rpm for 5 minutes to obtain the cell pellet. The pellet was suspended in 3.0 ml of a diluted RP-30 preparation whose concentration was minimal absorbance at 280 nm that will give 100 % of the dead cells in the standard method, and incubated with shaking at 37°C or at 0°C. After being incubated for 15 and 30 minutes, the suspension was centrifuged at 1,000 rpm for 5 minutes and the cytotoxicity of the supernatant was tested. As control, 3. Oml of the RP-30 preparation alone was incubated at 37°C or 0°C, centrifuged, and the cytotoxicity of the supernatant was assayed. Adsorption

of the cytotoxic factor on FM3A, a cell line derived from the mammary tumor of C3H mice (kindly supplied by Dr. H. Okano of Kyushu Cancer Center), was also examined by the same method.

Effect of colchicine on the cytotoxicity

Suspension of E-cells provided with PBS(-) containing 0.1 mM of colchicine was preincubated at 37°C for 30 minutes to destroy the intracellular microtubular structure. One hundredth ml of the suspension was mixed with 0.5 ml of RP-30 preparation diluted to have the absorbance from 1.0 to 0.2 (about 30 to 100 times dilution) with PBS(-) containing the drug at the same concentration, and then incubated at 37°C for 2 hours.

Inhibition of the cytotoxic activity by sugars

One hundredth ml of E-cell suspension provided with PBS(-) or VBS was mixed with 0.5 ml of RP-30 preparation containing sugars at a concentration from 10 μM to 10 mM, and incubated in the same way. The inhibitory effect of sugars was estimated by lowering of the cytotoxicity at a minimal absorbance that gave 100% of the dead cells under the standard condition.

Binding of the cytotoxic factor with D-glucose

NP-30 preparation was diluted to have the absorbance 0.832 (30 times dilution) with PBS(-) containing 0.1 mM of n-glucose, incubated at 37°C for 30 minutes with shaking and centrifuged at 45,000 rpm for 1 hour with a RP55 rotor. A decrease of n-glucose content in the supernatant was estimated by phenol-H_2SO_4 method. Content of sugar in the supernatant of the NP-30 preparation diluted 30 times with PBS(-) was also determined as a blank test. One ml of NP-30 preparation of the absorbance 13.44 was dialyzed against 2 L of distilled water for 3 days with 6 changes of water and lyophilized, then the absorbance was converted to the weight.

RESULTS

The results of the cytotoxicity test under various conditions are shown in Fig. 1. As given in the figure, the cytotoxicity was not evoked at 0°C under both successive shaking and 20 minute-intervals' shaking. Even at 37°C, less than 30 % of the cytotoxicity was evoked when shaken at 20 minute-intervals.

As shown in Fig. 2, the percentage of the dead cells increased almost proportionally with the incubation time.

The effect of the divalent cations on the cytotoxicity is shown in Fig. 3. The result indicates that the cations do not affect the cytotoxicity of RP-30 preparation.

Fig, 4 shows the result of the adsorption test at 37°C. The cytotoxic factor in RP-30 preparation was completely adsorbed on the target cells within 15 minutes at this temperature. Moreover, FM3A cells adsorbed the factor within 15 minutes at 37°C. Also at 0°C E-cells could adsorb the cytotoxic factor on their surface, but the rate of adsorption was seemed to be slow **(Fig. 5)**.

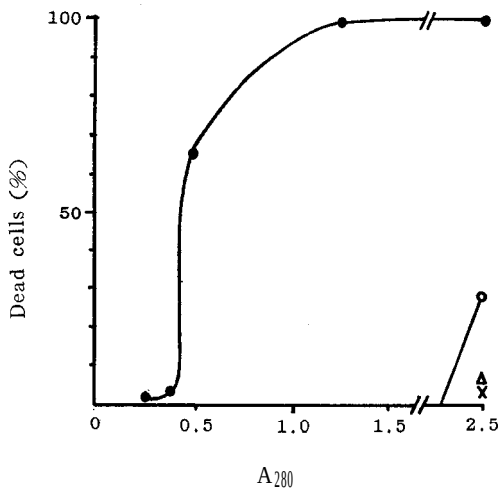

Fig. 1. Effect of temperature and shaking on cytotoxicity of R-I-a. (●): 37°C with successive shaking. (o) : 37°C with shaking at 20min.-intervals. (△) : 0°C with successive shaking. (×) : 0°C with shaking at 20min.-intervals.

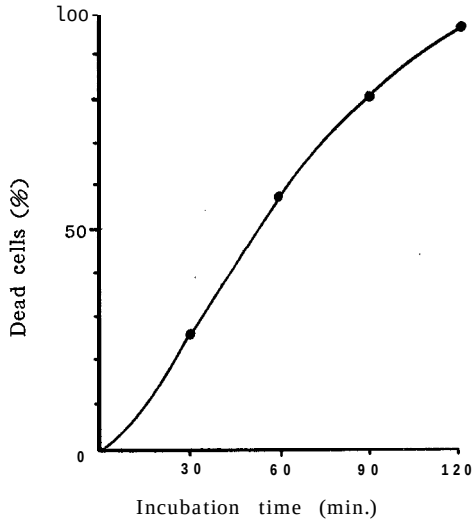

Fig. 2. A time course study as to cytotoxicity of RP-30.

The cytotoxicity was fairly blocked by an inhibitor of microtubules, colchicine as shown in Fig. 6. The colchicine-treated E-cells could, however, adsorb the factor on their surface within 15 minutes at 37°C as shown in Fig. 7.

The inhibitory effect of various sugars on the cytotoxicity of RP-30 preparation is shown in Table 1. The cytotoxic factor did not show so narrow specificity against inhibitory sugars. Additionally it was concluded that 1 μg of NP-30 preparation bound with 0.75 μg of n-glucose.

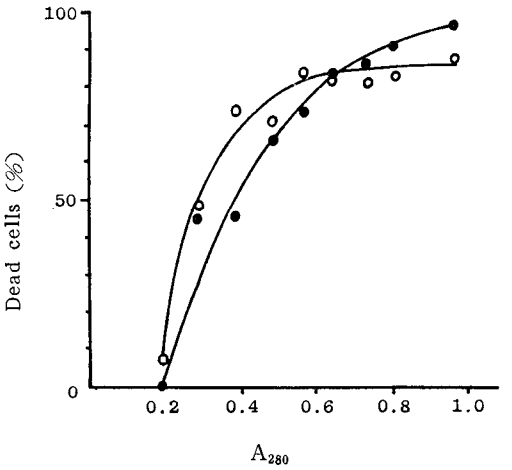

Fig. 3. Effect of EDTA on cytotoxicity of RP-30. Without EDTA (•), with 10mM EDTA (○).

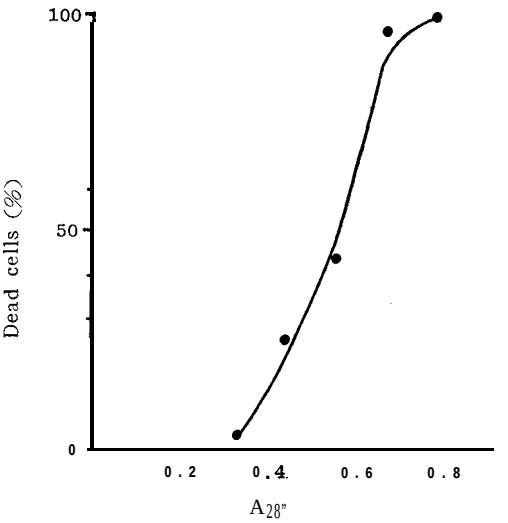

Fig. 4. Adsorption of cytotoxic factor on E-cells. -●- Original RP-30; -○- Preincubated with the cells for 15min.

DISCUSSION

In the preceding paper, it was reported that both the extract of normal spleens and that of the E-cell resistant mouse spleens had the cytotoxicity to the same extent (Momii et al., 1979). Elucidation of the mode of action of the cytotoxic factor in the mouse spleen extract was pursued mainly with the RP-30 preparation.

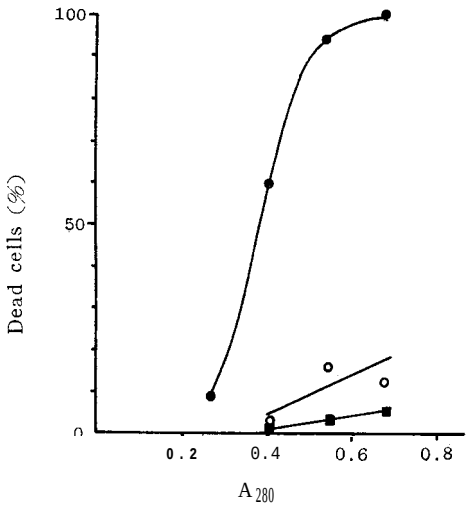

Fig. 5. Adsorption, of cytotoxic factor on E-cells at 0°C. Preincubated for 15min. (○), for 30min. (■), untreated RP-30 (●).

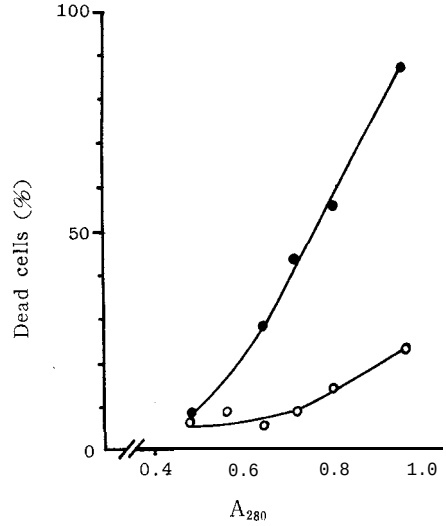

Fig. 6. Effect of colchicine on cytotoxicity of RP-30. Untreated E-cells (●), and colchicine treated cells (○).

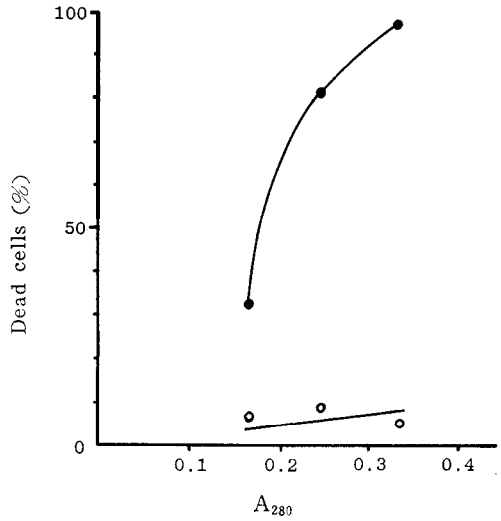

Fig. 7. Adsorption of the cytotoxic factor to colchicine-treated E-cells. -●- Untreated RP-30. -○- Preincubated with the cells at 37°C for 15min.

A display of the cytotoxicity was temperature- and shaking-dependent (Fig. 1). This fact implied that the shaking was to enhance a chance of binding of the cytotoxic factor with the target cells.

The cytotoxic factor was adsorbed onto the target cells at 37°C as well as at 0°C. As the cytotoxic activity was not evoked at 0°C and only a small

Table 1. Inhibitory effect of sugars on cytotoxicity of RP-30.

Sugar	Concentration (mM)			
	10.0	1.0	0.1	0.01
Glucose	4*	4	2	1
Mannose	4	4		
Galactose	2	1		
Fructose	4	4	4	1
Fucose	1			
Glucosamine	3	2	2	
N-Acetylglucosamine	1			
Xylose	1			
Ribose	1			
Glucuronic acid	1			
Maltose	4	2	1	
Cellobiose	1			
Sucrose	2			
Inositol	1			
Mannitol	1			
Tetrahydropyrane	1			

* 4: over 60% inhibition
3: 30-60% inhibition
2: 10~30% inhibition
1: less than 10% inhibition

number of the cells was killed in 15 minutes' incubation, the adsorption of the cytotoxic factor on the surface of the cells did not directly affect the death of the cells.

Furthermore, the colchicine-treated E-cells, which could also adsorb the cytotoxic factor, were less susceptible to the cytotoxic action. From these results, it is assumed that the cytotoxic factor is adsorbed on the surface of the target cells within 15 minutes and then transferred into the intracellular space through endocytosis mediated by a colchicine-sensitive structure, or microtubules. The cytotoxic factor which entered into the cytoplasm is expected to inhibit a metabolism in the target cells. Then, it results in the death of the cells. Microtubules are reported to be inactive at a low temperature (Roth, 1967; Rodriguez and Piezzi, 1968).

u-glucose showed a strong inhibitory effect on the cytotoxicity, whereas D-xylose or D-glucuronic acid showed no effect. This suggests that the hydroxymethyl group at 6-position of glucose is essential for the effect. n-fructose also inhibited the emergence of the cytotoxicity. Furthermore, the hydroxyl group at Z-position of glucose would also be significant for the inhibitory effect, because D-glucosamine and N-acetyl-D-glucosamine showed a weak or no inhibitory action. As D-mannose inhibited the cytotoxicity, it is suggested that both equatorial and axial configuration of hydroxyl group at 2-position of hexose ring are effective for the inhibitory action. n-galactose also inhibited the cytotoxicity slightly.

Mäkelä classified lectins into 3 groups by their specificity to inhibitory sugars as follows (Kawaguchi, 1976): (i) lectins inhibited by L-fucose, (ii) lectins inhibited by n-galactose, and (iii) lectins inhibited by n-glucose or

D-mannose. According to his classification, the cytotoxic factor in the spleen extract belongs to the class (iii). Disaccharides except maltose did not show a strong effect. Even, the effect of maltose was weaker than that of D-glucose. Why disaccharides do not so strongly inhibit the cytotoxicity as monosaccharides is not understood. The inhibitory action of the sugars would be due to preventing the cytotoxic factor from being adsorbed on the surface of the target cells as in the case of lectins, because the cytotoxic fraction could bind with D-glucose.

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