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A Cytotoxic Subcellular Fraction of Mouse Spleen Extract

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This paper deals with screening and partial purification of a cytotoxic substance from the spleen of mice resistant to the Ehrlich ascites tumor. The purification was performed by a fractionation with ammonium sulfate, sucrose density-gradient centrifugation, and isoelectric focusing electrophoresis. The cytotoxicity was recovered in the microsomal fraction of the spleen extract. Thus, it was concluded that the cytotoxic substance was not humoral.

In order to elicit tumor resistance in host animals, two methods have been employed mainly, that is, inoculation of attenuated tumor cells (Révész, 1960 ; Buzzi and Buzzi, 1974 ; Morgan and Eng, 1972; Kato ***et al.,*** 1972) and immunopotentiation by host-mediated adjuvants (Old ***et al.,*** 1961; Chihara et ***al.,*** 1970; Grohsman and Nowotny, 1972; Lindenmann, 1974; Kato ***et al.,*** 1973; Likhite and Halpern, 1973).

In research for tumor immunology, transplantation immunity with non-specific transplantable tumors has been adopted as a model of tumor immunity (Lindenmann, 1974).

A complex (MA: y-RNA), composed of methylated bovine serum albumin (MA) and yeast RNA (y-RNA), attenuated Ehrlich ascites tumor cells (E-cells) by being transferred into their cytoplasm by endocytosis (Maekawa and Kushibe, 1972). Mice inoculated with the attenuated tumor cells could reject the following challenges of the intact cells (Maekawa and Kushibe, 1969; 1972).

Killer T-cells and humoral mediators, lymphokines, released from the cells have been assumed to take an important role in cellular immunity which mainly contributes to tumor resistance (Mitchison, 1955; David, 1971). Furthermore, subcellular components which mediate the tumor resistance were also recognized in peripheral lymphoid tissues or lymphocytes (Clewell ***et al.,*** 1969; Fraser and Cater, 1972; Ferluga and Allison, 1975).

The present paper deals with screening and partial purification of a cytotoxic substance from the spleen of the E-cell resistant mice.

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MATERIALS AND METHODS

Cells and **Mouse**

E-cells were maintained in the peritoneal cavity of the ddN mouse which was supplied by the Animal Center at Kyushu University. One fifth ml of ascitic fluid containing the tumor cells (about 10^8 cells per ml) was transferred from mouse to mouse weekly. Other cells used were Sarcoma-180 cells, FM3A (cell line derived from mammary tumor of the C3H mouse), TBV (cell line from W/Fu rat leukemia) and normal ddN mouse spleen cells. FM3A and TBV, kindly supplied by Dr. H. Okano of the Kyushu Cancer Center, were cultured in RPMI-1640 supplemented with calf serum (Research Institute for Microbial Disease, Osaka University) up to 5 % in a sealed TD 40 tissue culture bottle (Ikemoto Rikaki, Ltd.) at 37°C. Normal mouse spleen cells *were pre*-pared as follows. Spleens of the ddN mouse killed by cervical dislocation were washed with VBS (veronal buffered saline) and minced finely with scissors. The minced tissues were further dissociated by the shearing force developed by 2 pieces of slide glass, suspended in 10 ml of VBS and allowed to stand in a test tube till blocks of the tissues fell down. The supernatant without the blocks was washed with VBS 3 times. All these procedures were carried out at 4°C.

Mouse resistant **to E-cells**

An MA: y-RNA complex was prepared as described previously (Maekawa and Kushibe, 1972). MA was purchased from the Miles Laboratories Inc. (Elkhart, Ind., U. S. A.) or was prepared by esterification of bovine serum albumin fraction V (Sigma Chemical Co., St. Louis, Mo., U. S. A.) by Fraenkel-Conrat's method (Fraenkel-Conrat and Olcott, 1945). Yeast RNA (Sigma Chemical Co.) was further purified by the method reported previously (Maekawa and Kushibe, 1964). To a female mouse 10^5 of E-cells incubated with 6.0 mg of the complex at 25°C for 20 minutes were inoculated intraperitoneally. The mouse was challenged 3 times with 2×10^5 of intact E-cells at 20 day intervals intraperitoneally.

Spleen extract and its fractionation

Spleens obtained from the mice resistant to E-cells and/or from normal mice of the same age were frozen and thawed several times. Then, the spleens were minced and further ruptured with the rotating homogenizer pestle in 2 volumes of PBS(-) (Ca^{2+}, Mg^{2+}-free phosphate buffered saline). The homogenate was centrifuged at 10, 000 rpm for 30 minutes with a IB rotor (Sakuma Seisakusho, Ltd.). The resulting precipitate was homogenized again in 2 volumes of PBS(-) and centrifuged under the same conditions. The supernatants (spleen extract) were put together and added $(NH_4)_2SO_4$ up to 30 % saturation. The resulting precipitate was collected by centrifugation, suspended in PBS(-) and dialyzed against the saline. The dialyzate was centrifuged at 10, 000 rpm for 30 minutes and cytotoxicity of the supernatant was assayed. The obtained fractions are referred to R-I-a from the resistant mouse spleens and N-I-a from the normal ones. Furthermore, the spleen

extract was ultracentrifuged at 45,000 rpm for 1 hour with a RP 55 rotor (Hitachi Seisakusho, Ltd.). The precipitate and the fluffy layer was homogenized in a small amount of PBS(-) and ultracentrifuged again. The precipitate was suspended in PBS(-) by homogenization and centrifuged at 10,000 rpm for 30 minutes. The resulting supernatant was added $(NH_4)_2SO_4$ up to 30 % saturation. The precipitating fraction was collected by centrifugation, suspended in PBS(-) and dialyzed against the saline. The dialyzate was centrifuged at 10,000 rpm for 30 minutes and the cptotoxicity of the supernatant was assayed. The obtained fractions are referred to RP-30 from the resistant mouse spleens and NP-30 from the normal ones.

Cytotoxicity test

E-cells harvested from the peritoneal cavity of the mouse were washed with PBS (−) or VBS several times and the concentration was adjusted to 8 $\times 10^7$ cells per ml. One-hundredth ml of the cell suspension and 0.5 ml of the spleen extract or its subfraction were mixed and incubated at 37°C for 2 hours with successive shaking in duplicate test tubes. Dead cells were stained with 1.5 ml of 0.4 % nigrosin solution in saline. Evaluation of the cell number was carried out on a Bürkel-Türk hematometer. Specific activity was represented as reciprocal of absorbance at 280 nm of the preparation which resulted in 50 % of dead cells. Total activity was given as a product of the specific activity and total absorbance (the absorbance times volume) of the spleen extract or its subfraction.

Sucrose **density gradient centrifugation**

On 0.5 ml of **60** % sucrose solution 27.5 ml of linear density gradient sucrose solution (from 35 to 5 %) and 2.0 ml of RP-30 or NP-30 preparation were layered in order. The sucrose was dissolved in PBS(-). Centrifugation was carried out at 25,000 rpm for 2 hours with SW 25.1 rotor (Beckman Instrument, Inc.). After centrifuged, the content in the centrifuge tube was divided into 40 fractions (0.75 ml in each fraction tube). The cytotoxicity of each fraction, whose absorbance at 280 nm was adjusted to 1.0, was assayed. The active region was arbitrarily divided into 3 subfractions (I, II and III).

Isoelectric focusing of the cytotoxic subfractions

The cytotoxic subfractions obtained by the sucrose density gradient centrifugation were dialyzed against 2 liter of 10 mM NaCl solution for 2 days with 3 changes of the solution. The dialyzates were filtered through a prefilter AP 25 type (diameter: 13 mm, Millipore Corp., Bedford, Mass., U.S.A.). The filtrates were subjected to isoelectric focusing at 1 to 2°C for 48 hours by Svensson's method (Vesterberg and Svensson, 1966). Anti-convection agents used were 50% sucrose or 60% glycerin solution. Concentration of carrier ampholyte (LKB, Stockholm, Sweden) was 1.0 %. After the separation was over, the content in the column was fractionated by 1.8 ml portion in each fraction tube. Absorbance at 280 nm and pH of each fraction were measured.

Enzyme-treatment of the cytotoxic fraction

The cytotoxic fraction RP-30 was treated with bovine pancreatic RNase A

or *Streptomyces griseus* protease (Sigma Chemical Co.). RNase A was dissolved in PBS(-) (10 mg per ml) and dialyzed against the saline overnight. The enzyme activity was determined by Koga's method (Koga et al., 1967). The protease was dissolved in PBS($-$) (20 mg per ml) and centrifuged at 10,000 rpm for 30 minutes. The supernatant was also dialyzed. The dialyzate was centrifuged again if necessary. The proteolytic activity was determined after Hagiwara (Hagiwara, 1961).

The enzyme solution (5×10^3 units and 1.3×10^4 units per ml of the protease and the RNase A, respectively) was mixed with an equal volume of RP-30 preparation and the mixture was incubated at 37°C for 30 and 60 minutes. After the incubation, the mixture was heated in boiling water for 5 minutes to inactivate the enzymes, and subjected to the cytotoxicity test. In the control experiment, RP-30 preparation was incubated at 37°C without the enzymes and then heated in boiling water. The cytotoxic factor did not alter its activity by heating in boiling water as shown in Fig. 1.

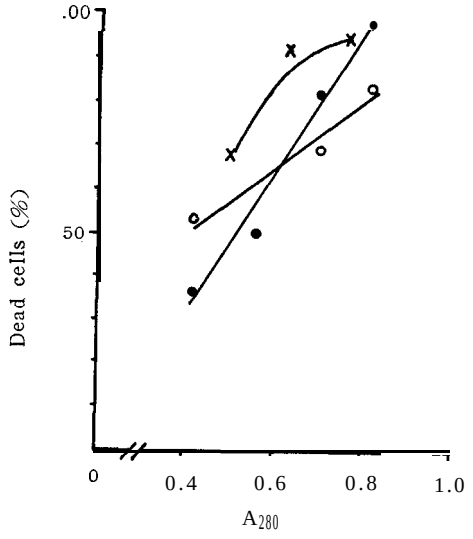

Fig. 1. Thermostability of cytotoxic factor.
RP-30 heated at 100°C for 5 (×) and 30 minutes (○). Untreated RP-30(●).

RESULTS

The cytotoxicity was recognized in both R-I-a and N-I-a preparations. Both had an equal extent of the cytotoxicity as shown in Fig. 2. However, the weight of one resistant mouse spleen was 0.18 g (average of 675 spleens), whereas that of one normal mouse spleen was 0.14 g (average of 511 spleens). So the total activity of R-I-a and N-I-a preparations obtained from one mouse was 26.1 and 18.6, respectively.

The specific and the total activity of the normal mouse spleen extract,

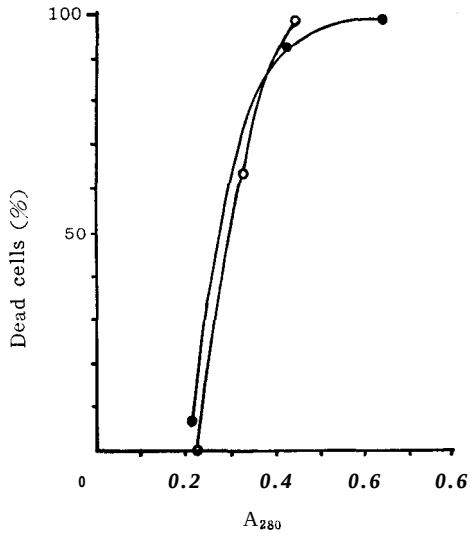

Fig. 2. Cytotoxicity of active fractions from spleens of mice resistant to E-cells, R-I-a (-●-), and those of normal ones, N-I-a (-o-).

Table 1. Cytotoxic activity of normal mouse spleen extract and its subfractions.

Fraction	Specific activity[1]	Total activity[2]
Spleen ext.	0.015 (1.0)[3]	10.8
Dialyzed spleen ext.	0.21 (14.0)	113.9
N-I-a	0.98 (65.3)	100.0

1) The reciprocal of absorbance at 280nm that gave 50% of dead cells.
2) The product of the specific activity and the total absorbance of the preparation.
3) The specific activity of the spleen extract was defined as 1.0.

and its active fractions were shown in Table 1. The spleen extract had so slight cytotoxicity that the intact extract could kill only about a half of the cells in the incubation mixture. However, when the extract was dialyzed against PBS(-) overnight, the specific and the total activity arose up to 14 and 10 times, respectively. It is, therefore, suggested that dialyzable substance(s) suppress the cytotoxicity in the spleen extract.

The cytotoxicity was recovered in the microsomal fraction of the spleen extract, so it is concluded that the cytotoxic substance is not humoral. This was confirmed by the sucrose density gradient centrifugation.

The result of the sucrose density gradient centrifugrtion is shown in Fig. 3. The relative activity was almost constant from the fraction number 10 to 29. Dilute sucrose solution less than 20 % in PBS(-) was not cytotoxic during the 2 hours' incubation. So, each fraction whose concentration of sucrose

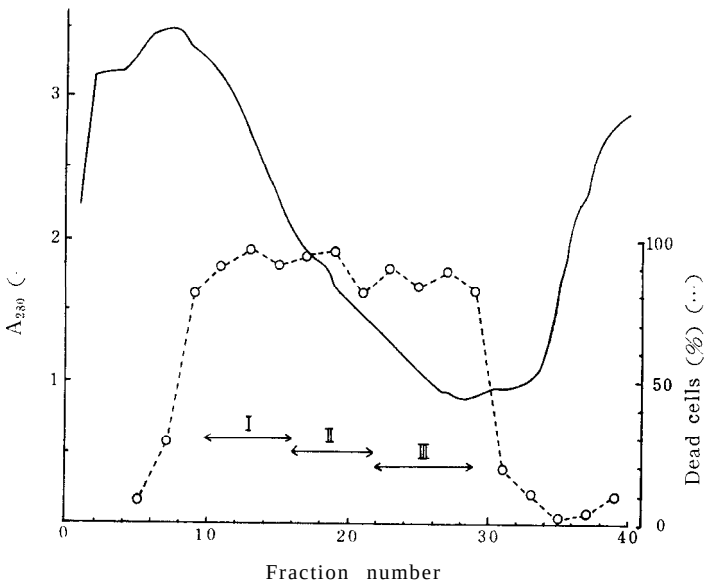

Fig. 3. Sucrose density-gradient centrifugation of the KP-30
Rotor : SW-25.1, Centrifugation : 25,000 rpm. 2 hrs., Sucrose density gradient: 35-5% (0.5 ml of 60% sucrose solution was laid at the bottom of the tube). 13 drops were collected in each fraction tube.

was diluted to less than 20% was subjected to the cytotoxicity test. Sedimentation constant of the cytotoxic substance was calculated to be 400 to 70 S by Martin's equation (Martin and Ames, 1961), when the partial specific volume of the sedimentating particle was provided to be 0. 725 cm^3 per g (Van Holde, 1975).

The isoelectric focusing patterns of the cytotoxic subfractions obtained by the sucrose density gradient centrifugation were shown in Fig. 4a-b-c respectively. The precipitate appeared near the position of pH 4.7 in all the subfractions and this part alone was active (indicated by dotted area). Although these subfractions (I, II, and III) were still impure, their amino acid compositions were preliminary estimated in order to acquire some clews to the further purification. The results were shown in Table 2 and in a topographic Chart (Fig. 5).

As seen from Fig. 5, their amino acid compositions were surprisingly close each other. Therefore, it is presumed that the active principles may be composed of cohesively similar unit.

The cytotoxic activity in RP-30 preparation was diminished by treatment with *Streptomyces griseus* protease, as shown in Fig. 6. However, RNase A did not alter the cytotoxic activity as shown in Fig. 7.

The inhibitory effect on transplantability of E-cells was observed in N-I-a preparation when a higher dose was administered (Maekawa *et al., 1978).* All the mice looked well through the experiment.

The cytotoxic fraction was also effective on not only E-cells but also

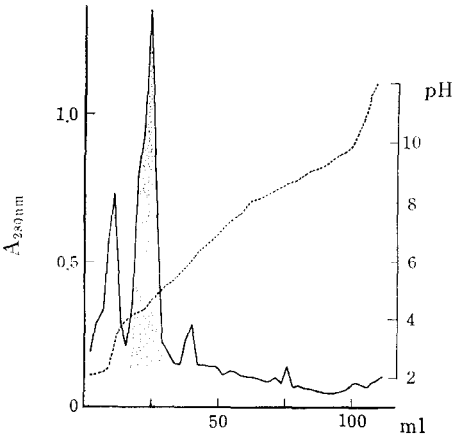

Fig. J-a. Isoelectric focusing of the fraction I. Anti-convection agent was 50% sucrose solution. Voltage : 230~780V ; Current: 4mA~1.4mA ; Sample charged, 16.5ml (total A_{280nm} =12)

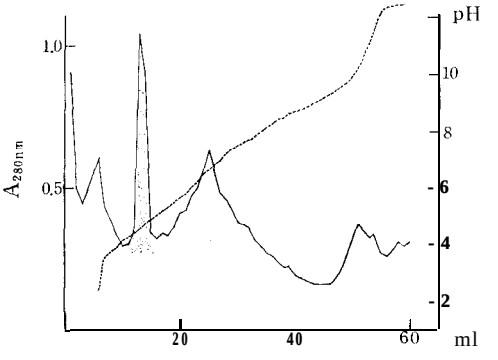

Fig. 4-b. Isoelectric focusing of the fraction II. Anti-convection agent was 60% glycerin solution.

other cells such as FM3A, TBV, Sarcoma-180 cells and normal mouse spleen cells (Table 3).

DISCUSSION

Factors that mediate various physiological phenomena in the field of tumor immunology are actively being investigated by many workers. Especially, the presence of cytotoxic substances is assumed to be concerned in host's tumor resistance (Meltzer and Bartlett, 1972; Vaillier et al., 1972; Luzzio, 1973). Although these substances were reported to be humoral, the cytotoxic factor in this investigation is found not to be humoral by the ultracentrifugation technique and the sucrose density gradient centrifugation. Ferluga reported

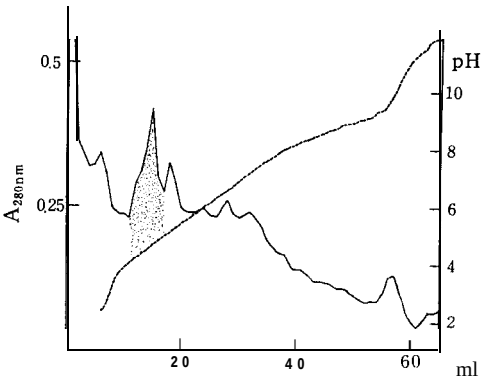

Fig. 4-c. Isoelectric focusing of the fraction III. Anti-convection agent was 60% glycerin solution.

Table 2. Amino acid composition of the fraction I

Amino acid	μ mole	μ g
Lys	0.0206	2.641
His	0.0074	1.015
Arg	0.0193	3.015
Asp	0.0303	3.458
Thr	0.0184	1.861
Ser	0.0225	1.960
Glu	0.0354	4.537
Pro	0.0158	1.535
Gly	0.0239	1.364
Ala	0.0276	1.962
Val	0.0202	2.003
Met	0.0005	0.066
Ilk	0.0151	1.709
Leu	0.0343	3.882
Tyr	0.0074	1.208
Phe	0.0133	1.958
Total (μg)		38.043
Recovery %		48.63

that the cell membrane fraction from oxazolone-activated lymphoid tissue had cell-injuring action which was not due to contaminant of the humoral lymphotoxin (Ferluga and Allison, 1975). Fraser and Cater (1972) showed that sedimentable subcellular components from lymphoid tissue of mouse sensitized with allogeneic tumor had a therapeutic effect against the same tumor in syngeneic host. The cytotoxic activity in this investigation may also be contributed by cell membrane fragments, which were obtained by homogenization of the spleen to be fragmented to 70 to 400 S particles.

The dose-response curve of the cytotoxicity had a steep slope. It is, therefore, assumed that certain amount of the active particle is necessary for the cell to be killed. The amount of the cytotoxic activity in a resistant mouse was 1.4 times higher than that in a normal one. This fact may indi-

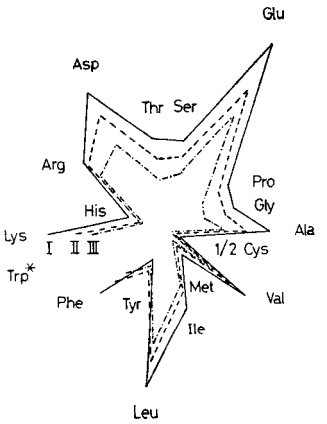

Fig. 5. Comparison of amino acid compositions of the fraction I, II, and III.
* remain to be estimated

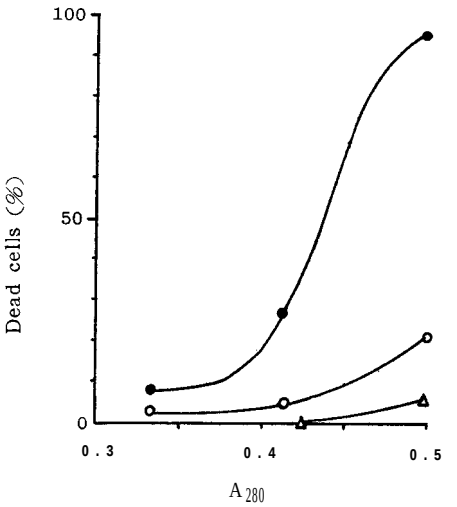

Fig. 6. Protease-sensitivity of cytotoxic factor.
KP-30 treated with protease from ***Streptomyces griseus*** at 37°C for 30 min. (○) and 60min. (△). Control (●).

cate that a certain amount of the cytotoxic factor is also necessary for the host to reject the tumor transplanted.

All the cytotoxic subfractions by the sucrose density gradient centrifugation were found to be similar to one another in the isoelectric focusing pattern. Especially, all of them contained a major component that was precipitated near the pH 4.7. The effluent from the column was fractionated into several portions and dialyzed against PBS(-) with 5 changes of the saline, and the cytotoxicity of the dialyzates was assayed. Only the fraction obtained

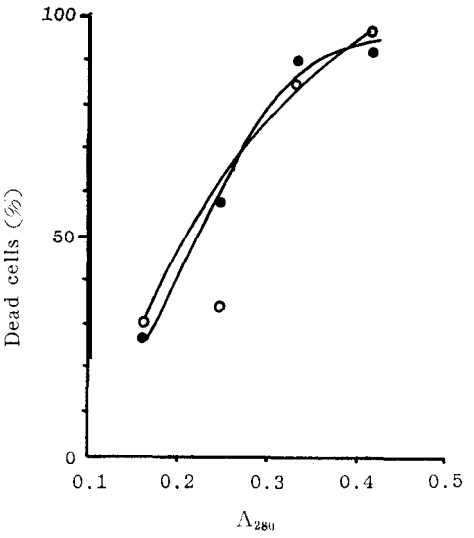

Fig. 7. Stability of cytotoxic factor against ribonuclease A.
RP-30 incubated with RNase A at 37 C for 60min. (○). Control (●).

Table 3. Cytotoxicity of RP-30 preparation on cells other than E-cells.

Cells	Conc. of RP-30[1]	Dead cells. (%)
Sarcoma-180	0.547	89. 1
	0.684	99. 5
FM3A	0.250	97. 8
Normal spleen cells	0.333	100
TBV	3.2 [2]	100

1) Represented by the absorbance at 280 nm.
2) At this concentration, 44.9 % of E-cells was died.

from the region near pH 4.7 could represent the slight activity (14.9% of dead cells with the absorbance 0.392 in the case of the subfraction II). When R-l- a and N-I-a preparations were dialyzed against McIlvaine's buffer (pH 5.0) for three hours, precipitate appeared in the dialyzate. The supernatant of the dialyzate showed no cytotoxicity. However, the precipitate, dissolved in 0. 1M Na_2HPO_4-NaOH buffer (pH 11.0) and dialyzed against PBS(-) for 24 hours with 3 changes of the saline, showed 1.3 times stronger activity than the parent fraction. Recovery of the total activity in the precipitate was 95 %. No lowering of the cytotoxicity was recognized when the cytotoxic preparation was dialyzed against 0.1M Na_2HPO_4-NaOH buffer (pH 11.0) for 6

hours. As the isoelectric focusing system in this experiment contained a slight of NaCl, the exact isoelectric point of the components was not determined. From these results, however, it is assumed that the isoelectric point of the cytotoxic factor is near 4.7.

The cytotoxicity was affected by treatment with the protease from *Streptomyces griseus*, but was not diminished by pancreatic RNase A. From these results, it is concluded that the cytotoxic activity is mediated by a protease-sensitive substance or protein.

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