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Cytotoxic Effect of the Culture Supernatant of *Clostridium tetani*

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The cytotoxic effect of the culture supernatant of *Clostridium tetani* on mouse leukemia L1210 cells was studied. As the cytotoxicity of the culture supernatant reached almost the maximum at 24 h after the start of cultivation of the organism, the supernatant fluid was fractionated by addition of ammonium sulfate and the fraction that showed the highest specific cytotoxic activity was subjected to gel filtration. The cytotoxic activity was separated in two peaks in parallel with the hemolytic activity; both activities were decreased by preincubation with cholesterol. An independently purified fraction of tetanolysin also exhibited cytotoxic activity. The results showed that one of the cytotoxic substances was tetanolysin, a hemolysin produced by *Clostridium tetani*. Furthermore, tetanolysin potentiated the cytotoxicity of 5-fluorouracil in combined treatment.

Keywords——*Clostridium tetani*; mouse leukemia L1210 cells; supernatant fluid; cholesterol; tetanolysis; 5-fluorouracil; combination effect

When spores of some nonpathogenic *Clostridia*, which are spore-former anaerobes, were injected into animals bearing a tumor or necrotic tissue, they germinated and grew selectively in the tumor or necrotic tissue without concomitant effect on normal tissue and exhibited an oncolytic effect.[1] It was suggested in the *in vitro* experiment that this effect was due to the action of a soluble substance released by the germinated spores.[2] On the other hand, Lapointe and Portelance[3] reported that an antitumor substance, which was effective in animals bearing a solid tumor, was present in the culture supernatant of *Clostridium perfringens* type A.

This report deals with the cytotoxic effect of the culture supernatant of *Clostridium tetani* on mouse leukemia L1210 cells.

Materials and Methods

Cultivation of *Clostridium tetani*——*Clostridium tetani* strain Harvard A-47 was kindly provided by Dr. S. Nishida, Department of Bacteriology, School of Medicine, Kanazawa University, Kanazawa. The organism, stocked in liver-liver broth, was inoculated into the same medium and cultivated at 37°C for 24 h. This procedure was repeated three times. The seed culture was prepared by three successive transfers of the culture fluid into TYG medium (pH 7.4) containing 3% trypticase peptone (BBL Inc.), 1% yeast extract (Difco Lab.), 0.5% sodium chloride, 0.5% glucose, and 0.1% sodium thioglycolate, with cultivation at 37°C for 24 h. The last seed culture was inoculated into TYG medium and cultivated at 37°C for various periods. The supernatant fluid was obtained by centrifugation of the culture fluid. The growth of the organism was measured by reading the absorbance at 570 nm as a measure of turbidity.

Purification of the Cytotoxic Substance——The culture supernatant (1 liter) obtained by cultivation for 24 h was fractionated by addition of ammonium sulfate and the fraction precipitated by ammonium sulfate at 30—50% saturation was dissolved in 30 mM phosphate-buffered saline (pH 7.4, PBS) and dialyzed against PBS. The fraction was then applied to a Sephadex G-100 column (2.1×55 cm) equilibrated with 50 mM phosphate buffer (pH 7.5) and eluted with the same buffer at 4°C. Fractions of 5 ml were collected at a flow rate of 10 ml/h. Tetanolysin was purified by ammonium sulfate fractionation, acetone precipitation, and repeated gel filtration according to the method of Mitsui *et al.*[4] The final preparation, though it was not preincubated with cysteine, showed an activity of about 4.5×10^4 hemolytic units/mg of protein.

Cell Culture and Assay of Cytotoxic Activity——Mouse leukemia L1210 cells were kindly provided by Dr. T. Ujiie, Cancer Research Institute, Kanazawa University, Kanazawa. The cells were serially cultured in RPMI 1640 medium containing 10% fetal calf serum (RFC 10 medium) in a CO_2 incubator at 37°C. The cells collected by centrifugation were suspended in Hanks' solution to a concentration of about 5×10^5 cells/ml and were treated with a test fraction at 37°C for 30 min, unless otherwise described. After the treatment,

the cells were collected and were resuspended to the original volume in RFC 10 medium. A portion (1 ml) of the cell suspension was taken into a test tube and cultured in a CO_2 incubator at 37°C for 24 h. Cell viability was measured by staining with 0.2% Trypan Blue solution.

Assay of Hemolytic Activity——Hemolytic activity was assayed by the method of Roth and Pillemer[5] with slight modification. The hemolysin added to a test tube was diluted to 3 ml with PBS and preincubated at 37°C for 15 min. Three ml of 1% freshly prepared guinea pig erythrocytes (washed several times and suspended in PBS) was then added, and the mixture was incubated at 37°C for 30 min. After centrifugation at 3000 rpm for 5 min, the hemoglobin content of the supernatant was determined by reading the absorbance at 545nm. One hemolytic unit (HU) is defined as that concentration of hemolysin which lyses 50% of the erythrocytes contained in the reaction mixture under the conditions described above. Cysteine, an activator of oxygen-labile hemolysins, was not added to the preincubation mixture, because it was toxic to L1210 cells.

Effect of Cholesterol on the Cytotoxic Activity——Cholesterol (Wako Pure Chemicals) was dissolved in 99% ethanol to give a concentration of 1×10^{-3} M and diluted homogeneously to various concentrations with Hanks' solution. Hemolysin (12 HU) was added to 2 ml of the cholesterol solution. After preincubation at 37°C for 30 min, 2 ml of L1210 cell suspension (about 10^6 cells/ml) was added to the solution and the whole was incubated in a CO_2 incubator at 37°C for 30 min. The cells treated were collected by centrifugation and were cultured as described above.

Measurement of Protein Concentration——Protein concentration was measured by the method of Lowry *et al.*[6] with bovine serum albumin (fraction V, Nakarai Chemical Co.) as a standard.

Combined Treatment——One ml of L1210 cells (about 5×10^5 cells/ml) suspended in RFC 10 medium was taken into a test tube and exposed to 5-fluorouracil (5-FU, Kyowa Hakko Kogyo Co.) with or without various amounts of hemolysin in a CO_2 incubator at 37°C for 24 h.

Results

We examined the cytotoxicity of the culture supernatant of *Clostridium tetani* on mouse leukemia L1210 cells. As shown in Fig. 1, the cytotoxic activity of the culture supernatant increased with the growth of the organism and reached a steady level between 24 and 72 h; subsequently it tended to decrease gradually. In these experiments, TGY medium itself was confirmed not to be cytotoxic. From these results, it was evident that cytotoxic substances were present in the culture supernatant of *Clostridium tetani*.

The culture supernatant obtained by cultivation for 24 h was fractionated by addition of ammonium sulfate and the precipitate formed was dissolved in PBS and dialyzed against PBS. This fraction was applied to a Sephadex G-100 gel column. The cytotoxic activity was eluted as two peaks in parallel with the hemolytic activity (Fig. 2). This result suggested that one of the cytotoxic substances might be tetanolysin and the other might be a high molecular hemolysin reported by Mitsui *et al.*[4] When tetanolysin was purified by the method of Mitsui *et al.*,[4] the cytotoxic activity was fractionated in parallel with the hemolytic activity. Fig. 3 shows that the two activities are eluted in the same fraction in the second gel filtration. Moreover, we examined the effect of cholesterol, an inhibitor of tetanolysin,[7] on the cytotoxic activity of the high molecular fraction (tubes 19—21) and the low molecular fraction (tubes 25—30). When the low molecular fraction was preincubated with cholesterol, the cytotoxic activity was decreased and indeed, it was completely inhibited by 10^{-6} M cholesterol (Fig. 4).

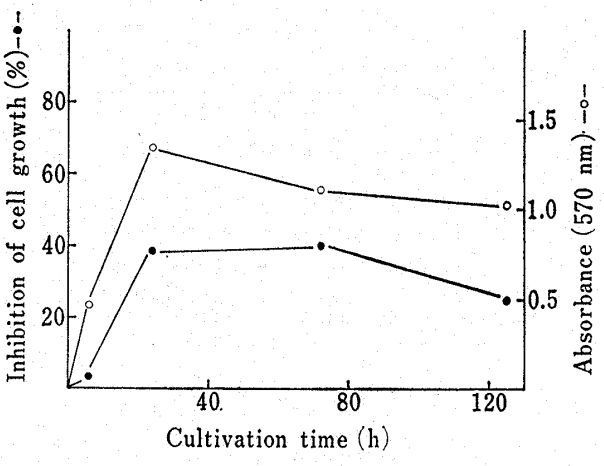

Fig. 1. Effect on Cell Growth of the culture Supernatant of *Clostridium tetani* cultivated for Various Periods

Ammonium sulfate was added to the supernatant fluid obtained by centrifugation of the culture fluid to give 70% saturation. The precipitate formed was dissolved in PBS and then dialyzed against PBS. The fraction (1.6 mg of protein) was added to 4 ml of cells (about 2.5×10^5 cells/ml) suspended in RFC 10 medium and was treated for 24 h as described in "Materials and Methods".

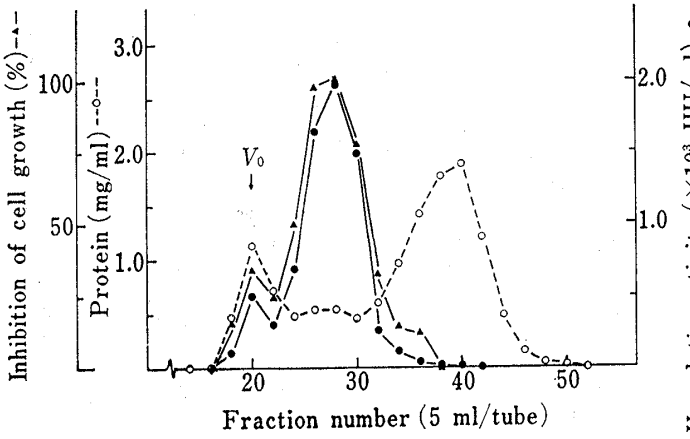

Fig. 2. Sephadex G-100 Column Chromatogram of the Fraction obtained by Ammonium Sulfate Precipitation between 30 and 50% Saturation

A solution (11.0 ml) containing 9.7×10^4 HU of hemolysin and 109 mg of protein was applied. Cells were treated with 2.5 μl of each fraction per ml. V_0 is the void volume.

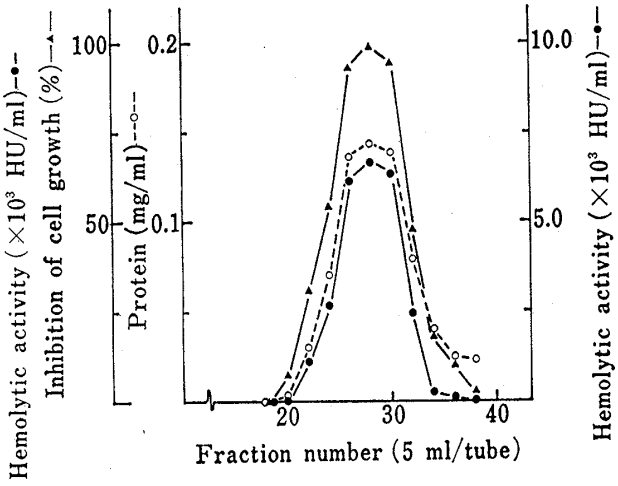

Fig. 3. Sephadex G-100 Column Chromatogram of the Tetanolysin Fraction obtained by the First Gel Filtration

A solution (18.0 ml) containing 12.5×10^4 HU of hemolysin and 5.1 mg of protein obtained by the method of Mitsui *et al.*[4] was applied. L1210 cells were treated with 0.25 μl of each fraction.

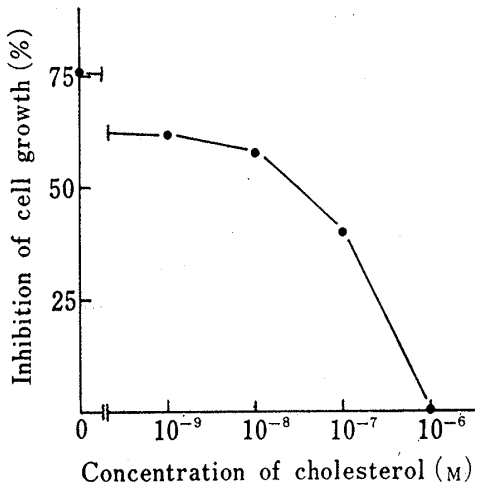

Fig. 4. Effect of Cholesterol on the Inhibition of Cell Growth by Tetanolysin

Tetanolysin (12 HU) was added to 2 ml of cholesterol solution. After preincubation at 37°C for 30 min, 2 ml of cell suspension (about 10^6 cells/ml) was added.

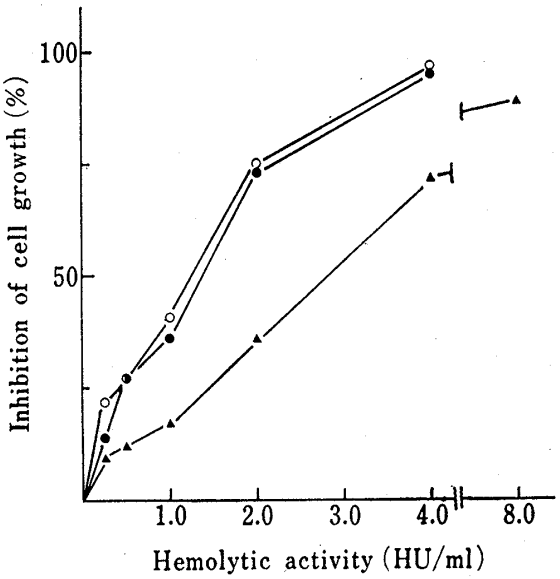

Fig. 5. Comparison of the Effects on Cell Growth of a High Molecular Hemolysin, Tetanolysin, and θ-Toxin

Cell suspension (about 5×10^5 cells/ml) was treated with the indicated amount of each hemolysin. ○: A high molecular hemolysin, ●: tetanolysin, ▲: θ-toxin.

The activity of the high molecular fraction was also inhibited (data not shown). From these results, we considered the low molecular hemolysin to be tetanolysin.

Further studies were carried out on the action of tetanolysin on L1210 cells. Fig. 5 shows the comparative cytotoxicity to L1210 cells of a high molecular hemolysin and tetanolysin produced by *Clostridium tetani* and θ-toxin, an oxygen-labile hemolysin like tetanolysin, produced by *Clostridium perfringens*. Two hemolysins derived from *Clostridium tetani* were cytotoxic to similar extents and about 1.3 HU per ml was required to induce 50% inhibition of the cell growth. θ-Toxin was less cytotoxic.

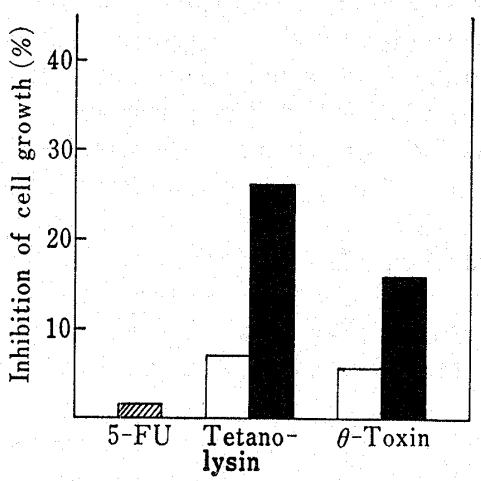

Fig. 6. Combination Effect of Tetanolysin or θ-Toxin with 5-FU on Cell Growth

Cells were treated with 5-FU (1 ng/ml, striped column), tetanolysin (10^{-3} HU/ml), or θ-toxin (10^{-2} HU/ml) alone (open columns), with 5-FU and each hemolysin (closed columns) for 24 h.

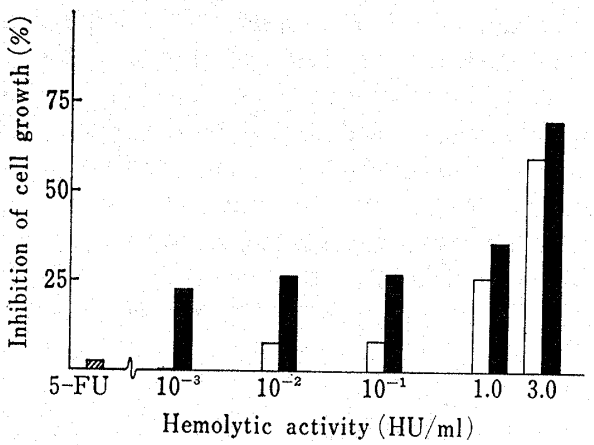

Fig. 7. Combination Effect of Various Amounts of Tetanolysin with 5-FU on Cell Growth

Cells were treated with 5-FU (1 ng/ml, striped column) or the indicated amount of tetanolysin alone (open columns), or with 5-FU and tetanolysin (closed columns) for 24 h.

It has been reported that some detergents,[8] vitamin A,[9] and polyene antibiotics,[10] which all cause cell lysis, potentiate the effect of antitumor agents. Therefore, studies were made to determine whether tetanolysin potentiated the cytocidal effect of antitumor agents. Fig. 6 shows that 5-FU (1 ng/ml) alone inhibited the growth of L1210 cells by about 1.6% and tetanolysin (10^{-3} HU/ml) alone did so by about 7%. On combined treatment with 5-FU and tetanolysin, the cell growth was inhibited by about 27.1%. θ-Toxin (10^{-2} HU/ml) also potentiated the effect of 5-FU, though the effect was less than in the case of combination with tetanolysin. When various amounts of tetanolysin were combined with 5-FU, smaller amounts of tetanolysin showed more potent combination effects than larger amounts (Fig. 7).

Discussion

Lapointe and Portelance[3] reported that the weight of solid tumor decreased and the survival time of the mice was prolonged when the culture supernatant of a low-virulence mutant of *Clostridium perfringens* type A was injected into mice bearing a solid tumor, and they suggested that the antitumor effect was due to the action of a soluble substance present in the culture supernatant of this organism. We therefore studied whether the cytotoxic substance was present in the culture supernatant of *Clostridium tetani*.

The cytotoxic activity of the supernatant fluid increased with the growth of the organism and reached almost the maximum at 24 h after cultivation. *Clostridium tetani* produces neurotoxin, hemolysin,[11] and proteases.[12] Among them, neurotoxin was reported not to be cytotoxic in the tissue-cultured cells.[13] When the culture supernatant obtained by cultivation for 24 h was applied to a gel column, the cytotoxic activity and hemolytic activity were eluted in the same fraction (Fig. 2). This cytotoxic activity was decreased by preincubation with cholesterol (Fig. 4) and fractionated in parallel with hemolytic activity during the purification of tetanolysin from the supernatant fluid of a 3-day culture. Furthermore, on electrophoresis of the tetanolysin fraction, the cytotoxic activity and the hemolytic activity were found in the parts of the gel corresponding to a major protein band (data not shown). Based on these results, we conclude that one of the cytotoxic substances in the culture supernatant of *Clostridium tetani* is tetanolysin. On the other hand, the activity found in the high

molecular region may derive from a high molecular hemolysin reported by Mitsui *et al.*[4]

Tetanolysin is an oxygen-labile hemolysin like streptolysin O, θ-toxin, and cereolysin, and it was reported to lyse erythrocytes, platelets, lysosomes,[14] or mycoplasma.[7] The binding site of these hemolysins is cholesterol in the cell membrane and the sensitivity of cells to them has been considered to be dependent on the content or the mode of existence of the membrane cholesterol.[7,15] Shinitzky and Inbar[16] reported that normal mouse lymphocytes contained about twice as much cholesterol as mouse ascites leukemia cells. On the other hand, other investigators[17] reported that lymphocytic leukemia cells contained more cholesterol than normal lymphocytes because of the lack of feed-back inhibition of sterol synthesis by extracellular cholesterol or because of an elevated sterol synthesis. Thus, it is possible that tetanolysin selectively acts on tumor cells, if they contain abundant cholesterol in the membrane.

Oxygen-labile hemolysins bind to cholesterol in the membrane and may change the membrane permeability. Polyene antibiotics, which bind to ergosterol or cholesterol in the cell membrane and lead to cell lysis, potentiate the activity of 5-FU and some other antitumor agents in combined treatment.[10c,d] We also studied the combination effect of tetanolysin with 5-FU. Tetanolysin potentiated the cytotoxicity of 5-FU by about 18.5% and θ-toxin, which was less cytotoxic than tetanolysin, did so by about 9.3%. The synergistic effect of amphotericin B with 5-FU has been shown to be due to an increase of the incorporation of 5-FU into cells.[18] Thus, tetanolysin may potentiate the effect of 5-FU in the same manner as other hemolytic substances.

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