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Partial Purification and Characterization of Cytotoxic Factors from Co-cultures of Macrophage Cell Line (J774 cells) with BLV-producing Cell Line

Shoshi INOOKA, Setsuko IWABUCHI*
and Kazuyoshi OKUBO*

Department of Animal Science, Faculty of Agriculture, Tohoku University, Sendai, Japan
**Department of Food Chemistry, Faculty of Agriculture, Tohoku University, Sendai, Japan*

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Summary

Cytotoxic activity, measured by the growth inhibition of C8 cells, was found in culture fluids from the cells (Co-J774 cells) which were obtained by the co-culture of J774 cells (macrophage cell line) with bovine leukemia virus producing fetal lamb kidney cells (BLV-FLK cells). Cytotoxic activity was highest after 4 days of culture, 1 day after the cells reached confluence. Cytotoxic activity of gel slices after non-denaturing polyacrylamide gel electrophoresis of the concentrated culture fluids from Co-J774 cells revealed two active bands: one in the front and the other corresponding to a molecular weight of around 68,000. Also, the fluids were chromatographed on Sephadex G-150, two active peaks, correspond to a molecular weight obtained using electrophoresis, were observed. One active band was obtained when the active samples, revealing low molecular weight, was chromatographed on thin layer.

Macrophages activated in vivo secreted a variety of products that lyse tumor cells such as toxic oxygen metabolites, arginase, protease, and tumor necrosis factor (TNF) and so on (1-4). However, cytotoxin production from these macrophages seemed to be limited for a few days during their incubation. Therefore, it was difficult to collect a large amount of cytotoxins and clarify their characteristics.

On the other hand, several cell lines of macrophage or macrophagelike cells which produce several cytotoxins have been established (5-8). Accordingly, several problems as to yield and activity may be settled by the adequate handling of these cell lines.

In earlier reports (9, 10), we tried to use the system of co-culture of macro-

phage cell line (J774 cells) with bovine leukemia virus producing fetal lamb kidney cells (BLV-FLK cells) (11, 12) and obtained the cell line secreting strong cytotoxin to C8 cells which are a clone of F81 cells. Moreover, we described that the cell line is a stable cell line which continuously produces cytotoxin which has cytotoxicity to several tumor cell lines in vitro (13).

The purpose of the present study was to partially purify the cytotoxic factors and clarify their characteristics.

Materials and Methods

Cells and cell cultures: Co-cultures of J774 cells with BLV-FLK cells were performed as previously described (9). Briefly, BLV-FLK cells which were grown in 25 cm^2 culture flasks and Dulbeccos' modified Eagles' mdium containing 10% fetal calf serum, 100 μg/ml streptomycine, 100 u/ml penicillin and 1.0 μg/ml fungizon (D-MEM), and after 3-4 days of culture, the medium was removed and J774 cells were added. After 4 days of co-culture, the cells which were removed by scraping were subcultured every 2-4 days. Here, for convenience, we named the cell line after co-cultures as Co-774 cell line. Thus, at present, Co-J774A and Co-J774B lines have been maintained for more than 150 passages since November of 1985.

Preparation of culture fluids for cytotoxic assay: Co-J774 cells in generations 102 were grown in 75 cm^2 culture flasks. After 4 days of culture, the culture fluids were collected and centrifuged for 10 min at $250 \times G$. Then, about 500 ml of culture fluid were freeze-dried. Again, they were dissolved in 50 ml of distilled water. These concentrated samples were used for the next purification.

Cytotoxicity assay: C8 cells were used as target cells. The cells were subcultured every 5-7 days in D-MEM. They were cultured at a rate of $2\text{-}4 \times 10^4$ cells per well in culture plate (24 wells, Falcon). After 1-2 days of culture, the samples were added to a well at 0.4 ml in final volume. They were incubated at 37°C for 1-5 days. Then, cell growth was examined under a phase contrast microscope and the cytotoic activity was assayed by measuring the dilution of the fractionated samples to inhibit 50% growth of target cells.

Chromatographic procedures: The concentrated samples (3 ml) were subjected to gel filtration on a Sephadex G-150 (2.0 by 120 cm) equilibrated in pH 6.9 buffer. All chromatographic procedures were performed at 4°C at a flow rate of approximately 12 ml/h. Approximately 8 ml of each fraction were collected. Fractions were divided into three groups, 1, 11 and 111 groups. Samples from each group were collected. They were freeze-dried and again resolved in D-MEM. They were tested in the cytotoxicity assay. The samples from group 111 were rechromatographed by a Sephadex CL-6B. Each fraction of approximately 1 ml was collected, and was tested in the cytotoxicity assay to C8 cells.

Also, the samples from each fraction were electrophoresized on slab-polya-

crylamide gels and analyzed by dencitometer.

Polyacrylamide gel electrophoresis : 2.7 ml of the concentrated samples were further reconcentrated in 0.5 ml and the 100 μl were electrophoresized on 12.5% discpolyacrylamide gels under non-denaturing conditions. To locate cytotoxic activities, the gel was cut into 0.5 mm thick slices, and proteins from each slice were eluted by the addition of 1 ml D-MEM or PBS. Then, the samples were centrifuged. The supernatant fluids were streillized by membrane filtration and these two fold dilutions were assayed for cytotoxic activity.

Thin layer chromatography : The samples in group 111 were chromatographed on thin layer (silicagel, Wakogel-B5) and colored with sulfate. The part of the gel was cut into four groups and suspended in 2 ml D-MEM. After elution from gel, they were centrifuged and strilized by membrane filtration. Then, the cytotoxicity was tested. Also, the samples which were fractionated by non-denaturing conditions polyacrylamide gel electrophoresis were spotted on thin layer.

Results

Cells characteristics and cytotoxin production from Co-J774 cells : In a previous report (13), we described the production of cytotoxin from Co-J774 cells in generations from 19 to 130 and also, showed that the characteristics of Co-J774 cells in cell growth, phagocytosis, and morphology are similar to those of J774 cells. In the present study, the characteristics of Co-J774 cells in generations from 131 to 150 were examined. In general, production of cytotoxin and cell growth were not altered. Namely, the Co-J774 cells grew at a rate of $6\text{-}8\times10^5$/ml after 4 days when the cells were cultured at a rate of $1\text{-}2\times10^5$ cells/ml. Cytotoxicity was found in the culture fluids of Co-J774 cells after 3 and 4 days of culture. Also, the cytotoxic activity was found to be continuous and stable in the culture fluids of Co-J774 cells in generations from 131 to 150. No cytotoxicity was found in the culture fluid of J774 cells which were cultured at a rate of $1\text{-}2\times10^5$ cells/ml.

Gel filtration analysis of the cytotoxins : The fluids were subjected to gel filtration on Sephadex-G 150 column and approximately 3 ml of each fraction was collected. Then, the fractions from No 10-No 20, No 21-No 29, and No 30-No 45 were pooled. The pooled solution was freezed-dried. Again, the substances were dissolved in the D-MEM and the cytotoxic activity was tested to C8 cells. The cytotoxic activities were observed in Fraction 11 and 111. No activity in fraction 1 was observed (Fig. 1a).

Next, the freezed-dried sample from No 111 was again subjected to gel filtration on a Sepharose CL-6B. Then, cytotoxicity in each fraction was tested. As shown in Fig 1b, strong cytotoxic activities were observed in Fraction 56-59. No cytotoxicity was found in other fractions (Fig. 4). On the other hand, bovine

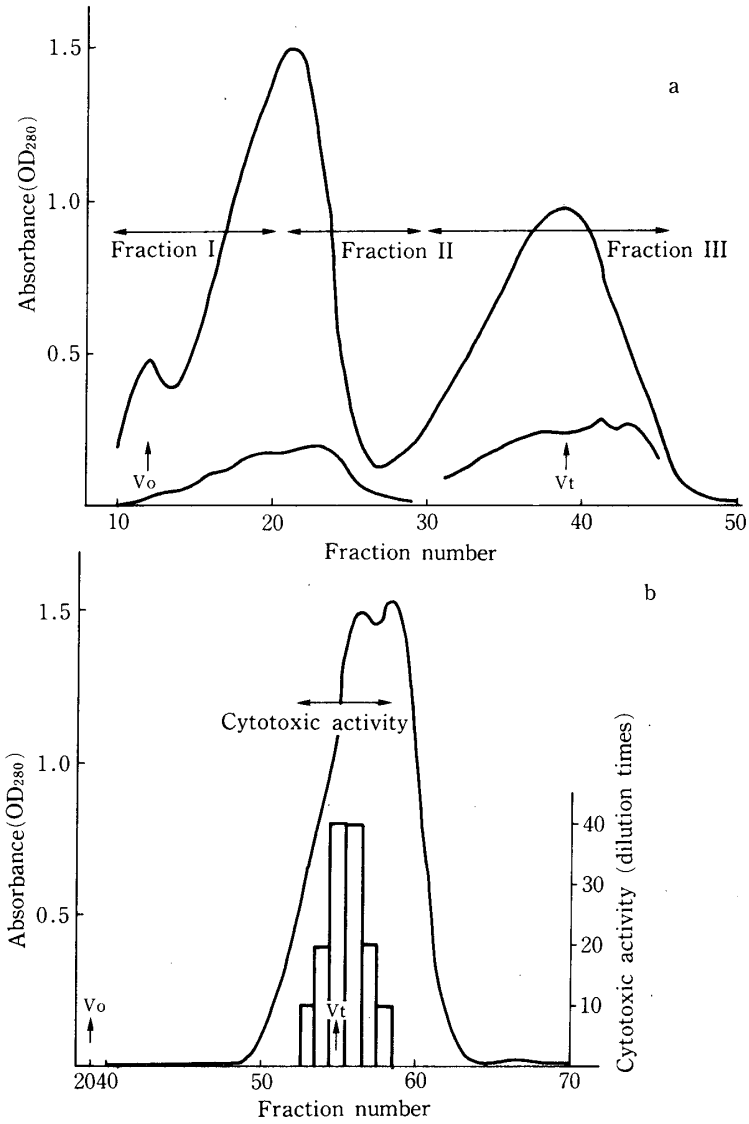

FIG. 1. Elution pattern of the concentrated culture fluids and cytotoxic activities after Sephadex G-150 and Sepharose CL-6B chromatography. The culture fluids from Co-J774 cells (3 ml) which were concentrated about 10 times, containing BSA (10 mg) and DNP-Ala (2 mg), were applied on the Sephadex G-150 (2.0×120 cm) and 8.0 ml of each fraction were collected. Each fraction in fractions 1, 11 and 111 was pooled and lympholized. The cytotoxic activity was tested, using the solution in which each fraction was again resolved in D-MEM (2 mg/ml and 10 mg/ml). In this concentration, no activities were observed in fraction 1, while they were observed in fractions 11 of 111. Also, the half in fraction 111 was again on Sepharose CL-6B column and 1.0 ml of each fraction were collected. The cytotoxic activities were revealed by the dilution times of each solution to inhibit 50% growth of C8 cells.

serum albumin and DNP-aranine which apply as a marker was observed in fractions 11 and 111, respectively.

Also, the samples (about 10 μl) from fractions 1, 11 and 111 were

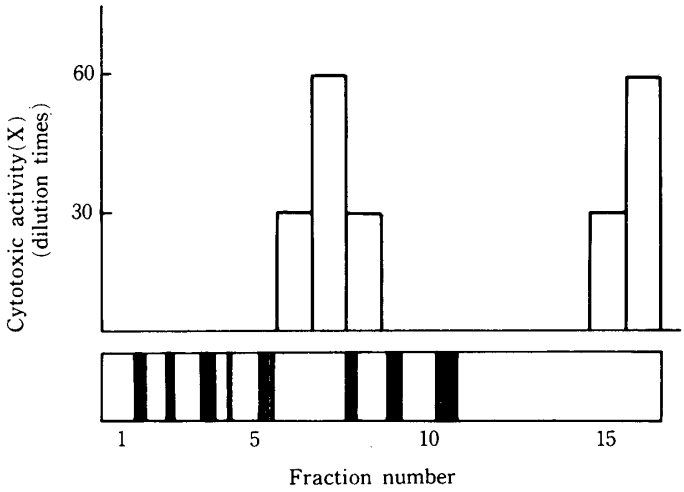

FIG. 2. Distribution of the concentrated culture fluids and the two cytotoxic activities zones after electrophoresis. The culture fluids (100 μl) from Co-J774 cells which were concentrated about 60 times were electrophoresized under non-denaturing conditions. The gel was cut into 0.5 mm thick slices and the substances which were contained in the gel were eluted in the 1 ml D-MEM. The cytotoxic activity was revealed by the dilution times of each solution to inhibit 50% growth of C8 cells.

electrophoresized on slab-polyacrylamide gels. Gel electrophoresis from 1 and 11 revealed a complex mixture of proteins which was located around bovine serum albumin, whereas no proteins were detected in gel electrophoresis from fraction 111 (Data not shown).

Acrylamide gel analysis of the cytotoxins involved in the culture fluids from Co-J774 cells: In order to clarify the characteristics of the cytotoxic fractions secreted by Co-J774 cells, the supernatant of culture fluids was obtained by centrifugation and was concentrated by lympholization. Gel electrophoresis of these concentrated fluids revealed a complex mixture of proteins (Fig. 2). Analysis of their cytotoxic activities after elution from the gel matrix gave two separate bands of cytotoxic activities. One of the bands was detected in the gel 2-3 cm from the top and another was involved 7-8 cm from the top. The molecular weights which were estimated by the acrylamide gel of molecular weight standards are found to be around 68,000 in the former and below 15,000 in the latter.

Thin layer chromatograms: The samples from No 56-No 59 in which the cytotoxic activities in gel filtration were found were spotted on the thin chromatography (silicagel, Wakogel-B5). The four bands were found (Fig. 3) and the cytotoxicity was found in the fourth bands (Fig. 4). Similar bands were obtained when the samples from No 16 which separated in acrylamide gel electrophoresis were spotted.

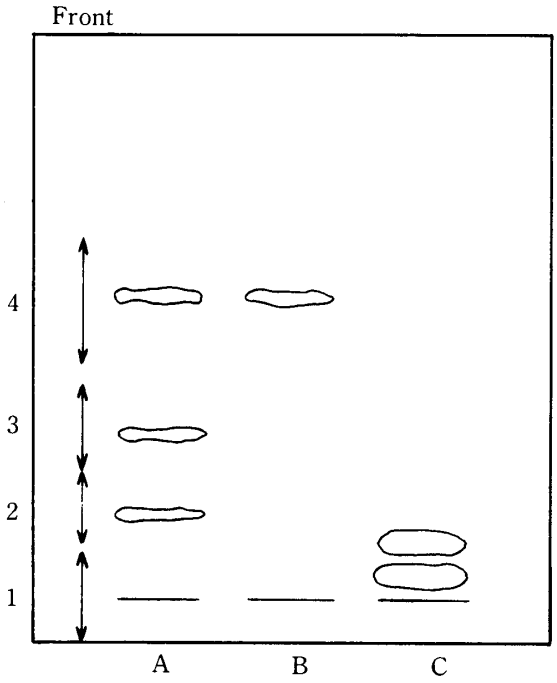

FIG. 3. Thin-layer chromatogram of the fractionated culture fluids containing cytotoxic activities. The gels (silicagel, Wakogel-B5) were chromatographed by solvent (chloroform, metanol, water) and colored with sulfate. A : The samples pooled from No 56-No 59 which were fractionated on Sepharose CL-6B (indicated in FIG. 1). B : The sample in No 16 which was fractionated by acrylamide gel electrophoresis (indicated in FIG. 2). C : The sample in No 7 which was fractionated by acrylamide gel electrophoresis (indicated in FIG. 2). The gel in A was cut into four groups and suspended in 2 ml D-MEM. The quantitative cytotoxic test was carried out by the direct addition of the fluid to C8 cells.

Discussion

Macrophages activated a variety of cytotoxic substances. However, it is very difficult to purify these products from the primary cultured macrophages because they secrete only a small amount. Previous study demonstrated that the cell line secreting strong cytotoxin to C8 cells was obtained by the co-culture of J774 cells with BLV-FLK cells. Also, it suggested that J774 cells were activated by BLV and permanently produced cytotoxic factors (9). Moreover, we have reported that the cytotoxic substances have a wide spectrum of tumor cells killing and were concentrated by lympholization (13). In our present study, it was demonstrated that the substances contain at least two different cytotoxic components. Namely, by both methods of gel filtration and polyacrylamide gel electrophoresis, it seemed to indicate molecular weights of around 65,000 for one component and of near DNP-alanine for another.

On the other hand, several macrophage cell lines, including J774 cells, produced complex cytotoxins. Aksamit and Kim (8) demonstrated that two

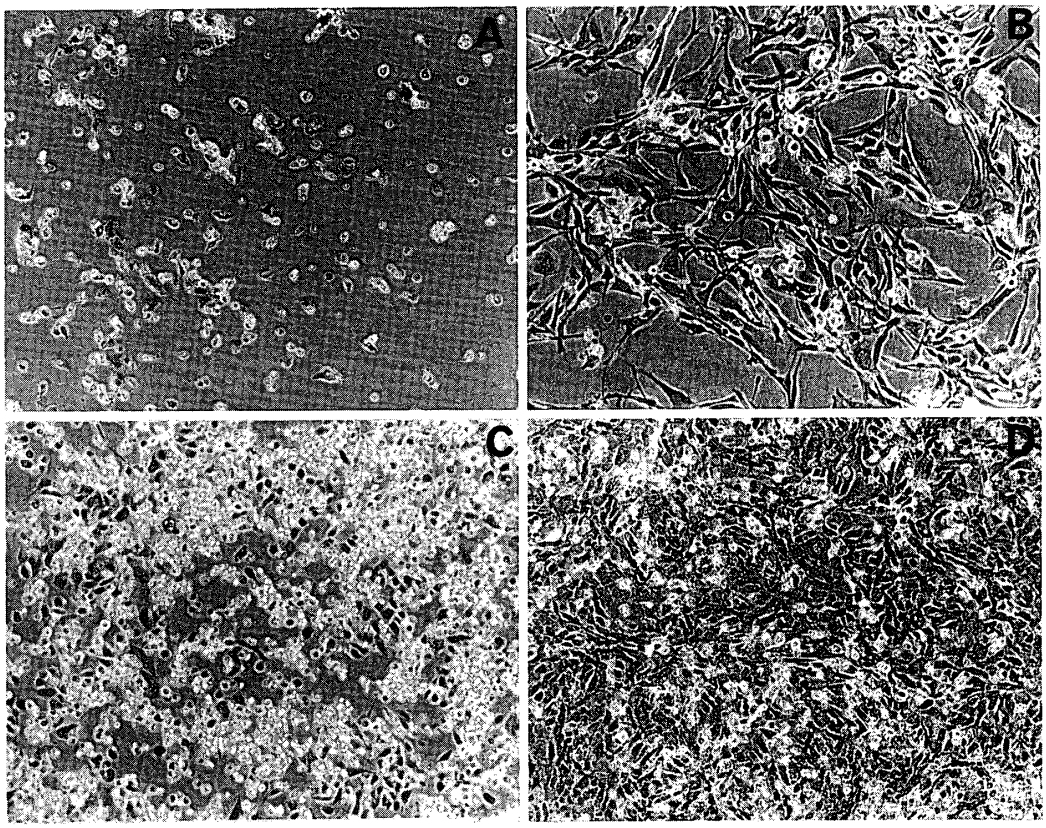

FIG. 4. Phase microscopic appearance of C8 cells. C8 cells were plated. Then, the fluids diluted 10 times which were fractionated by the Sepharose CL-6B (indicated in FIG. 1), and the fluids desolved in 2 ml D-MEM which were fractionated by the thin-layer chromatogram, were added. Considerable growth inhibition can be observed in the case of the addition of the fluid from No 57 in the Sepharose CL-6B (FIG. 4A) and from No 4 fraction in A in the thin layer (FIG. 4C), while no inhibition was observed in the case of the addtion of the fluid from No 60 in the Sepharose CL-6B (FIG. 4B). and from No 3 fraction in A in the thin layer (FIG. 4D). ×75

active factors were obtained from P388D1 cells : One in the void volume and the other cprresponding to a molecular weight of 40,000 to 50,000 by Sephadex G-200 chromatography. Also, Mannel, *et al.* (4), reported that macrophage-like tumor cells, PU5-1, 8 which stimulated with LPS, released tumoricidal factor and they gave the molecular weight to be 50,000 to 60,000.

In J774 cells, Kull and Cuatrecasas (14) demonstrated that J774 released necrosin with the molecular weight of 15,000 by SDS/PAGE and 60,000-50,000 by gel filtration. Also, Sakurai, *et al.*, (15) demonstrated that J774 cells after stimulation with TPA and LPS produced cytotoxin identical to TNF, with a molecular weight of 39,000 by gel filtration and 18,000 by SDS/PAGE.

In the present study, we demonstrated that Co-J774 cells produced two different cytotoxic factors without any stimulation. One of them may be TNF, because it was demonstrated that the culture fluids from Co-J774 cells revealed

the cytotoxicity to TNF sensitive cell line, L-M cells (by Dr. Haranakas' courtesy, unpublished data). On the other hand, it was demonstrated that the molecular weight of TNF is a range from 35,000 to 68,000. Accordingly, the cytotoxic factor, corresponding to molecular weight of around 68,000 in the culture fluids from Co-J774 cells may be TNF. Also, our previous data revealed that the culture fluids have about 3 times stronger cytotoxic activities to C8 cells than to L-M cells. Also, the present results from gel filtration, polyacrylamide gel electrophoresis and thin layer chromatography suggested that molecular weight of another cytotxin in below 10,000. Accordingly, one of them may be different from TNF.

These two cytotoxic factors will be further characterized, with particular attention to their differences in sensitivities to various target tumor cells. Also, it would be interesting to study the mechanisms of the activation of macrophages by clarifying those of the tytotoxic factor production from Co-J774 cells.

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