ANTIBODY FORMATION FOR MALIGNANT TUMOR.
I. ANTITUMOR ACTIVITY OF THE REGIONAL LYMPH NODE PROTEIN

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Synopsis

Lymphoid cells in regional lymph nodes of mice bearing Ehrlich ascites cell tumor have some cytotoxic action on cultured Ehrlich ascites carcinoma cells. The regional lymph node protein was fractionated on Sephadex G-200 column and on DEAE-Sephadex A-50 column. The antitumor activity was determined by cell culture test. The antitumor activity was mainly present in the fraction with β–mobility from DEAE-Sephadex A-50 column chromatography. The sedimentation coefficient of this protein was 6.5S. This protein was soluble and permeable through a cell-impermeable diffusion chamber. The minimum protein concentration required for suppression of tumor cell growth was about 300 µg for 10⁶ cultured JTC-11 cells in 10 ml culture vessel. In electrophoresis, this fraction moved mainly to β–position, and contained a small amount of α–band. The antibody in regional lymph node was loaded mainly on β– and α–mobility protein.

INTRODUCTION

Recently, some hypotheses have been advanced for antibody formation in malignant tumor, using immunological concepts and cellular contacts, through surface recognition factors. However, these hypotheses are provisional and we are still so ignorant about the basic mechanism. Antibody in lymphoid cells and in spleen cells has been exhaustively studied from the standpoint of cellular antibody by means of tissue culture techniques. Furth and Kabat,6) and Furth, Schuit, and Hijmans7) reported the immunoglobulin fraction in human lymph node and spleen. Dresser2) also reported the antibody formation by lymphoid tissue of guinea pigs in vitro. Hara9) described the immunological aspects for cancer, and observed that the regional lymphoid cells obtained from the mouse bearing Ehrlich ascites tumor have some inhibitory effect on the cultured Ehrlich ascites cells. Tanaka27) reported that this inhibitory factor is present in the supernatant of the regional lymph node homogenate, and mentioned some physical characters of this supernatant.

In the present work, the soluble fraction in the regional lymph nodes of the mouse bearing Ehrlich ascites tumor was fractionated on Sephadex G-200 and DEAE-Sephadex A-50 column, and the antitumor activity of each fraction was determined by a cell culture technique.

EXPERIMENTAL

Materials  Hybrid albino dd mice weighing about 20 g were used. These mice were inoculated subcutaneously with 5 × 10⁶ cells of Ehrlich ascites tumor cells or JTC-11
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Both kinds of cells were donated by the Cancer Institute of Okayama University Medical School. The mice bearing Ehrlich ascites tumor were sacrificed on the 10th day after the transplantation. The cervical and axillary lymph nodes were extirpated and were homogenized with 20 volumes of 0.01M phosphate buffer (pH 7.2) containing 0.01M NaCl in a glass-Teflon homogenizer. The homogenate was centrifuged at 100,000g for 50 mins. in a Hitachi Preparative Ultracentrifuge, Model 40P, and filtered through a Millipore filter (HAWP 025 00 25ea, ha 0.45 μ) to remove the floating fatty layer and particles. The filtrate was concentrated to about 5 ml in a small size Visking tube by ultrafiltration. These procedures were carried out at 2～5°. The protein concentration was determined by the absorbance at 280 mμ and by the colorimetric

![Graphic of gel filtration on Sephadex G-200]

**Fig. 1.** Gel filtration of supernatant on Sephadex G-200

A 2-ml supernatant solution of regional lymph node homogenate was applied on a 1.5 × 120 cm column, after dialysis against 0.01M phosphate buffer containing 0.5M NaCl (pH 7.2). Eluate was fractionated into 5-ml fractions by Toyo Autorecording Fraction Collector, Type S.F., 200 A. 32P activity was determined by 2-μ Gas-flow Counter fixing 0.2 ml in each 5 ml fraction.

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<tr>
<th>Table I. Phosphorus Compounds in Lymph Node</th>
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<td>Radioactivity (cpm)</td>
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<td>Normal</td>
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The lymph nodes were obtained from cervical and axillary portions of 10 mice 24 hrs. after the injection of 0.1 μCi Na32P04. The specimens (each 100 mg wet weight) were weighed, homogenized, and centrifuged. The supernatant was treated as described by Schneider, and the results are expressed by cpm/100 mg of wet lymph nodes. The lymph nodes of tumor-bearing mice (regional) were about 20～30% larger and heavier than those of the normal.
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method of Lowry et al., and nitrogen was determined by the micro-Kjeldal method. 
Phosphorus Compounds The lymph nodes were obtained from cervical and axillar portions of 10 mice 24 hrs. after injection of 0.1 mCi Na$_2$H$_5$PO$_4$, in order to recognize the nucleic acid fraction and acid-soluble substrate in the eluates of gel filtration (Fig. 1), and for the purpose of comparing the amount of phosphorus compounds between normal and regional lymph nodes (Table I). The specimens were weighed, homogenized, and centrifuged. The supernatant was treated as described by Schneider. 

Gel Filtration Gel filtration experiments aimed at fractionating the extract of lymph node were performed on Sephadex G-200 (AB Pharmacia, Uppsala) according to the method of Flodin and Killander. Columns of 4 x 80 cm and 1.5 x 120 cm were equilibrated with 0.01M phosphate buffer (pH 7.2) containing 0.5M NaCl, and were run in a cold room at 2°C. The elution rate was 5 ml/hr. The effluent fraction was concentrated by ultrafiltration in a small-sized Visking tube, or was lyophilized after desalting with Sephadex G-25 or absorbed on DEAE-Sephadex A-50 column with the same ionic strength as the initial buffer.

Ion-exchange Chromatography DEAE-Sephadex A-50 medium grade. AB Pharmacia, Uppsala) in the columns, equilibrated with 0.01M phosphate buffer (pH 7.2) containing 0.01M NaCl, was used throughout these studies. After dialysis of the original sample in the initial buffer, elution was started and was followed by an increasing linear gradient of NaCl in the same buffer at the constant pH of 7.2. The elution flow rate was about 20 ml/hr. Protein concentration of the fraction was determined by absorbance at 280 mÅ.

Electrophoresis Electrophoresis on cellulose acetate (oxoid) was used for the examination of the eluates from Sephadex G-200 column and the fraction from the DEAE-Sephadex A-50 column according to Kohn. 

Cell Culture Test The 1 x 10$^4$ Ehrlich ascites cells or JTC-11 cells were cultured in 10 ml of the YLE medium containing 20% bovine serum. The samples obtained from gel filtration or ion-exchange chromatography were exhaustively dialyzed against 0.01M phosphate buffer (pH 7.2) containing 0.05M NaCl, filtered through a sterilized Millipore filter, and 1 ml of the sample was added to the cell culture. Cell counts were taken on the Ehrlich ascites cells surviving 24- and 48-hr. culture at 37°C. Prior to counting, the cells growing on the wall of culture vessel were gently scraped off with a rubber cleaner. For counting the cell number, a method modified by Katsuta after Sanford's principle was employed, i.e., the number of stained nuclei was computed on the calculating plate of Bürker-Türk. For the determination of viability of the cells the unstained cell count method of Schrek was employed, i.e., after supravital staining with 1% Eosin Y solution, the number of stained cells was subtracted from the whole cell number counted previously.

RESULTS

The gel filtration experiments gave the results shown in Fig. 1. The ionic strength of the eluant was increased by using 0.01M phosphate buffer (pH 7.2) containing 0.5M NaCl, instead of the Tris-HCl buffer (pH 7.2) containing 0.2M NaCl, in order to minimize protein-protein interaction. The experiments were highly reproducible and were evidenced by electrophoresis on cellulose acetate of the eluted fractions. The
eluted fractions corresponding to the electrophoretic patterns were pooled and concentrated into four parts, called A, B, C, and D. Part A contained three components which moved to the cathode side in electrophoresis, with $\gamma$, $\beta_1$, and $\beta_2$ mobilities. Radioactivity of this part was high, as seen in Fig. 1, but depended on the nucleic acid fraction present. Part B contained components with $\beta_1$ and $\alpha$ mobilities. Part C contained $\alpha$, $\beta_2$, and albumin. Part D contained low-molecular weight substance of lipid-containing material and acid-soluble substrate, and passed through a collodion bag (Sartorius Membranfilter, Kollodiumhülsen, Göttingen) by ultrafiltration, but did not move from the original point on electrophoresis. The counts of $^{32}$P are also given in Fig. 1 and the high count of Part D depended on the acid-soluble substrate. Phosphorus

![Graph](image1)

**Fig. 2.** Antitumor activity of A, B, C, and D fractions obtained by gel filtration

The protein concentration in the middle portion of each fraction was adjusted and 1 ml each was added for cell culture test. Protein concentration was about 500 $\mu$g/ml.

![Graph](image2)

**Fig. 3.** Chromatography of supernatant on DEAE-Sephadex A-50

A 5-ml sample was applied on a 1.5 x 30 cm column. Elution was done with a linear gradient from 0.01 to 0.3M NaCl in 0.01M phosphate buffer of the same pH. A 300-ml mixing chamber was used. The last fraction was obtained by elution with 0.1N NaOH in 0.5M NaCl.
component of the supernatant solution was determined according to the method of Schneider26) and the results are given on Table I. Significant difference was not observed between normal and regional lymph nodes.

The results of cell culture test of these fractions are shown in Fig. 2. The parts B and C were effective in inhibition of the growth of JTC-cells in that order.

Chromatogram on DEAE-Sephadex A-50 column is shown in Fig. 3 and electrophoresis of the eluted peaks is also given in the same graph. These peaks were named I, II, III, and IV. The cell culture test of these peaks is shown in Fig. 4. Peak II was effective in suppressing the growth of JTC-cells, electrophoretically migrated in β-globulin region, and contained a small amount of protein with α–mobility. The aliquot test

![Fig. 4. Antitumor activity of I, II, and III fractions by DEAE-Sephadex A-50 chromatography](image)

The protein concentrations of peaks I, II, and III were adjusted by the optical density, and about 400 µg/ml protein was added for cell culture test.

![Fig. 5. Antitumor activity of peak II](image)

Protein concentration of peak II was 1000 µg/ml, and was added by diluting to 1 ml in the cell culture vessel.
of this protein is shown in Fig. 5. The minimum protein content of $\beta$ fraction required for the suppression of the cultured JTC cells is about 300 $\mu$g/ml for $10^4$ JTC-cells in 10 ml cell culture vessel. Ultracentrifugal analysis of peaks II and III is given in Fig. 6.

**DISCUSSIONS**

Efforts have been made for a long time to discover the immunogeneity and antibody formation for malignant tumor but the results were far from the aim. Weiler$^{30}$ reported that the tissue-specific antigen in particle fraction of rat liver homogenates disappeared in liver cancer of rat induced by feeding 4-(methylamino)azobenzene. Vogt$^{28}$ found that this antigen localized in the membranes of the endoplasmic reticulum. Nairn et al.$^{20}$ concluded that the antigen was detectable by the immuno-diffusion test in agar gel using microsomes and specific antiserum. Friedrich-Freksa et al.$^5$ reported that the antigen purified with Sephadex G-200 column was about 9S protein.

Finally, one may speculate that such an organ specificity is lost in transplantable or chemically induced tumor and a new cellular antibody for tumor cell may appear. Indeed, Klein$^{13}$ mentioned that there was a difference in the type of cellular antigen between a tumor induced by virus and that induced by a chemical agent. Maisin$^{16}$ found that the microsomal fraction of liver cancer had a tumor-specific antigenicity. Hara$^9$ reported that the cellular antibody for Ehrlich ascites tumor existed in the lymphoid cell of regional lymph nodes, and antitumor activity was most elevated on the 10th day after the tumor transplantation. Tanaka$^{27}$ observed the behavior of this lymphoid cells in Ehrlich ascites cell culture by the time-lapse micro-cinematography for 48 hrs., and found that lymphoid cells seemed like attacking the cancer cells, contacting and aggregating to the surface, the cancer cells were standing in pause, and moreover some cancer cells were broken, as if they were destroyed (killed). Mitchinson$^{17}$ and
Rosenau also observed that lymphocytes in the regional lymph node near the tumor-transplanted portion were aggregating to the surface of transplanted cells and destroyed in cell culture vessels. Forrester et al. reported the changes in electrophoretic mobility of tumor cells treated with antibody. These phenomena are dependent on the surface recognition theory. These facts seem to suggest some possibility that the animal host should be able to form a resisting or inhibitory factor against cancer in the body.

In the present experiment, the cellular antibody for Ehrlich ascites cell carcinoma was studied. The nature of the suppression factor for JTC-11 cell treated with Sephadex G-200 showed a globulin-like mobility in electrophoresis. DEAE-Sephadex A-50 chromatography separated the globulin components clearly, and the antibody for tumor cells was mainly involved in the II fraction (β-mobility in electrophoresis). Sedimentation coefficient of this protein was 6.5S.

It is interesting to note that the minimum effective protein concentration is about 300 μg/ml in the cell culture test, which usually needed at least 10^4 JTC-11 cells at the start in 10-ml cell culture vessel. The maximum effect was presumably from the cell culture test to be about 500~1,000 μg protein/ml as shown in Fig. 5. It is unknown whether this β-mobility protein is an immunoglobulin itself or not for the JTC-cells, because Ehrlich ascites cells and dd mice are homologous, but at least, the antitumor activity or cytolidal function was recognizable. It is also interesting that the antitumor activity in JTC-11 cell culture test was effective without the addition of complement, and generally, the complement substance is toxic to cell culture.

Recently, Hashimoto et al. reported the inhibitory effect of carcinostatic agents on antitumor activity of sensitized lymphoid cells. They observed that the lymphoid cells, when placed in a cell-impermeable diffusion chamber, were not effective for the target cells outside the chamber. This is probably due to the low protein concentration of the effective cytoidal factor, as was stated later. Sensitivity of the cells to the cytotoxic effect has been shown by the Möllers to depend on the density of the relevant antigen on the cell surface and "resistant" cells could be made sensitive by increasing the number of reacting antigenic receptors on the cell surface. They also suggested that immunization of a host lymphocytes to make the cell having compatible antigen might merely change the surface property of host lymphocytes to make the cells specifically adhere to the target cells. As they have elucidated, subsequent cytoidal action of the lymphoid cells may be mediated by a non-immune recognition process of antigen in compatibility, leading the target cell to death.

There are some possible mechanisms in which the protein of regional lymph nodes (containing the sensitized lymphoid cells) may inhibit the tumor cell growth such as (a) a cytotoxic antibody fixed to the lymphoid cells acts to destroy the tumor cells, (b) the sensitized lymphoid cells may attack the tumor cells by their phagocytic activity, and (c) cytoidal substance may be soluble because it passes through the diffusion chamber as suggested by the fact that the growth of 10^4 JTC-cells was inhibited when placed with 10^6 sensitized lymphoid cells enclosed in a cell-impermeable diffusion chamber (but was not suppressed by 2×10^5 cells).

The present experiment clarified the presence of cellular antibody, which was conceived from the fact that the sensitized lymphoid cells were aggregating on or destroying JTC-cells, and that it existed in β-mobility protein, partly in α-mobility.
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