# Acta Medica Okayama

# Antitumor factors of draining lymph node cells of the mouse sensitized with ehrlich ascites tumor cells. I. Antitumor effect of subcellular factor

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# Antitumor factors of draining lymph node cells of the mouse sensitized with ehrlich ascites tumor cells. I. Antitumor effect of subcellular factor*

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## Abstract

The regional lymph node cells of the mice sensitized with Ehrlich ascites tumor cells is known to possess a substance that shows antitumor activity on target cells (JTC-II cells). For the purpose to clarify the localization of this substance the regional lymph node cells from such sensitized mice were treated with trypsin solution of different concentrations (1.0 %, 0.2 %, and 0.01 %), and the tissue culture was carried out with JTC.II cells. As a result it was found that these lymph node cells lost antitumor activity. Next, by the differential contrifugation of these sensitized lymphocytes we obtained F1 fraction (700 g, sediment), F2 (8,500 g sediment), F3 (100,000 g sediment) and F4 (100,000 g supernatant). In the presence of each of these fractions tissue culture was conducted with JTC-II cells as target cells, and it was found that the substance with antitumor activity is contained abundantly in F2 fraction (8,500 g sediment) and F4 fraction (100,000 g supernatant). After giving due consideration to the results of these two experiments and also to the available data in the literature, we assume that the substance with antitumor activity is contained in the cell membrane component.

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# ANTITUMOR FACTORS OF DRAINING LYMPH NODE CELLS OF THE MOUSE SENSITIZED WITH EHRLICH ASCITES TUMOR CELLS

## I. ANTITUMOR EFFECT OF SUBCELLULAR FACTOR

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There is no doubt that the lymph node cells of the host play an important role as a cellular antibody in the homotransplantation immunity.

Klein (1), Hanaoka (2), Hara et al. (3) have demonstrated that the lymphocytes of the host sensitized by the transplantation of tumor cells show antitumor activity against the tumor cells both *in vitro* and *in vivo*. Further, by using two diffusion chamber sytem, Hara noted that the sensitized lymph node cells inhibit the proliferation of tumor cells even without coming in contact with the tumor cells. This indicates that anti-tumor substance, released from the lymph node cells, passes through the Millipore membrene of the diffusion chamber and acts on the tumor cells. Study on the problems of such cellular factors as to where such an anti-tumor substance is localized, whether it is localized in the cell membrane of it is released from the intracellular structure, would be important in the elucidation of cellular antibody.

On the basis of Hara's experiment the present study was carried out to locate such cellular antibody by isolating subcellular fractions from the draining lymph node cells of the mouse sensitized with Ehrlich ascites tumor cells. This paper describes the results of such a study as well as offers some opinions on the available literature relevant to this problem.

## MATERIALS AND METHODS

*Animals:* The animals used were Cb mice of about two months old weighing about 20 g, bred in the Okayama University Mouse Colony. The mice were fed on solid feed of Oriental Yeast Company and water was given *ad libitum*.

*Tumor cells:* The cells were Ehrlich ascites tumor cells maintained successively through the peritoneal cavity of Cb mice at Department of Pathology, Okayama University Cancer Institute (Dr. J. Satoh, Director).

*Culture cells:* The cells used were derived from Ehrlich ascites tumor cells

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and registered as JTC-11 cells at Japan Culture Association and maintained at
Okayama University Cancer Institure. HAMAZAKI (4) has demonstrated that
this strain of cells is capable of reproducing original tumors in mice.

*Sensitization:* To Cb mice weighing about 20 g, $5 \times 10^5$ of Ehrlich ascites
tumor cells were injected subcutaneously on the back between the scapulas.

*Trypsin treatment:* Two weeks after the sensitization lymph nodes from 10
mice were removed from axilla and neck, cut into small pieces, washed suffici-
ently with GKN solution three times to remove serum, and sensitized lymph
node cells are prepared. Next, trypsin-GKN solution is prepared in concentra-
tions of 1 %, 0.2 % and 0.01 %, each of these solutions is added to the sen-
sitized lymph node cells, and incubated for 45 minutes at 38°C. After the
incubation, an equal volume of the YLE solution is added to the trypsin-cell
suspension in each test tube and left standing for 10 minutes in a warm room
to inactivate trypsin. After the inactivation the cell suspension is centrifuged
at 2,000 rpm for 5 minutes. The supernatant is discarded and the sediment is
washed in the GKN solution twice, and by adding 1 %, 0.2 % and 0.01 %
trypsin solutions cell suspensions are prepared again. These treated lymph node
cells showed the survival rate of about 90 % as proven by Eosin-Y staining.
Identical procedures are carried out with normal mice and the lymph node cells
so prepared serve as the control group.

*Fractionation:* The sensitization is carried out by the above method on 20 Cb
mice, 2 weeks after the transplantation of tumor cells the regional lymph nodes
are taken out, cut into small pieces in Hanks solution, washed thoroughly,
Hanks solution is added to make the final volume 10 ml, these are homogenized
by a glass homogenizer in an ice-bath, the pestle is moved up and down 5 times
while revolving the pestle at a high speed, these are again placed in a homo-
genizer of Potter-Elevhjem type, and the pestle with Tefron head is gently
moved up and down 7 strokes to prepare the homogenates. Similar procedures
are carried out with normal Cb mice and the homogenates thus prepared serve
as the control gruop.

These homogenates are subjected to the centrifugation according to Schnei-
der's method. For the ultracentrifugation a Hitachi centrifuge is used and the
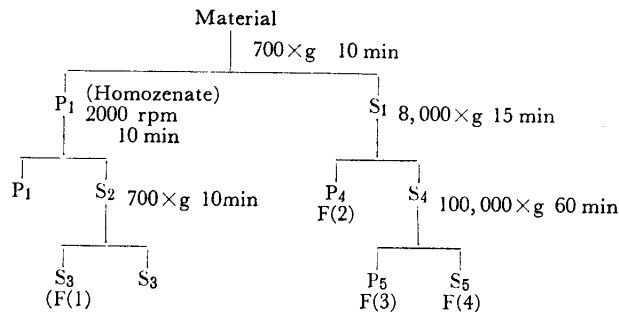rotor is 40 p.

Fig. 1  The Method of Fractination by Ultracentrifugation

*Step 1:* Each of these cell suspensions is centrifuged at 700 g for 10 minutes, the sediment is resuspended in Hanks solution, the suspension is centrifuged for 10 minutes at 2,000 rpm, and the supernatant so obtained is further centrifuged at 700 g for 10 minutes and the sediment thus obtained is taken as Fraction 1 ($F_1$).

*Step 2:* The supernatant in Step 1 is further centrifuged at 8,500 g for 15 minutes and the sediment separated is taken as Fraction 2 ($F_2$).

*Step 3:* The supernatant in Step 2 is subjected to ultracentrifugation at 100,000 g for 60 minutes, and the sediment separated is taken as Fraction 3 ($F_3$), and its supernatant as Fraction 4 ($F_4$). All centrifugation is conducted at 5°C and each fraction is stored at 5°C.

*Tissue culture method:* The culture medium used is composed of the mixture of YLE solution and inactivated bovine serum (8:2, v/v). To a test tube containing the fraction and JTC-11 cells 10 ml medium is added, after mixing well 1.5 ml each of the mixture is then put separately into a short culture vessel, which is placed at an angle of 5° in the incubator kept at 37°C, and the stationary culture is carried out for 24 and 48 hours.

1) Lymph node cells treated with 1 %, and 0.01 % trypsin are mixed with JTC-11 cells in proportion of $2 \times 10^3$ cells/ml : $2 \times 10^1$ cells/ml, respectively, and cultured.

2) Each of $F_1$, $F_2$, $F_3$ fractions prepared in Steps 1 to 3, that is the sediment remaining at the bottom of the test tube, is suspended in YLE solution, and this YLE suspension is again mixed with bovine serum in proportion of 8:2 (v/v) and to this medium JTC-11 cells (15,000 cells/ml) are added and cultured. In the case of the supernatant fraction ($F_4$), 2 ml supernatant, 6 ml YLE solution and 2 ml bovine serum are mixed and to this mixture 15,000 cells/ml of JTC-11 cells are added and cultured.

*The method of cell count:* The cell counts are taken once after 24-hour culture and 48-hour culture. This is done by decanting gently the medium from the culture vessel, and adding 1.5 ml crystal violet solution, the cells attached on the vessel wall are stained by leaving the vessel standing for 30 minutes at 3, °C, and after removing JTC-11 cells from the vessel wall by a rubber policemen and numbers of stained nuclei of JTC-11 cells are counted with Bürker-Turk hemocytometer. The average of three vessels is taken as the cell count each time.

### RESULTS

1) *Trypsin treatment :* The sensitized lymph node cells treated with trypsin solution at any of these concentrations lose their antitumor activity and either at 24-hour culture or at 48-hour culture their effect on the proliferation of target cells hardly differs from that of the control gruop as shown in Fig. 2.

2) *Subcellular fractions :* As illustrated in Figs. 3—6, $F_2$ shows a slight inhibitory effect and $F_4$ a marked effect on the proliferation of the target
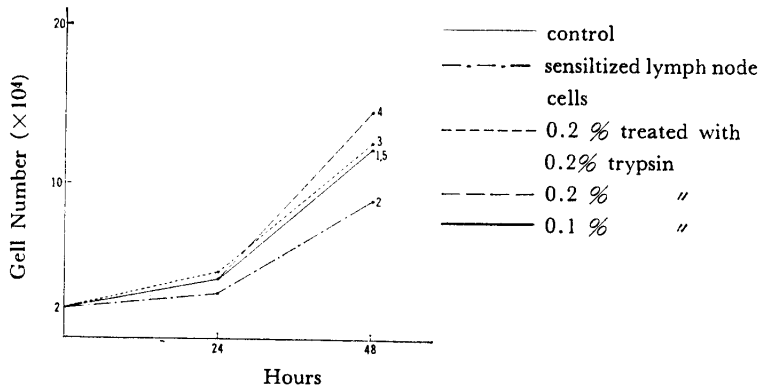
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Fig. 2  Inhibitory effect of trypsin-treated lymph node cells on
the proliferation of JTC-11 cells
    1)  control: only JTC-11 cells
    2)  mixed with the sensitized lymphoid cells
    3)  mixed with lymph node cells treated with 0.01 % trypsin
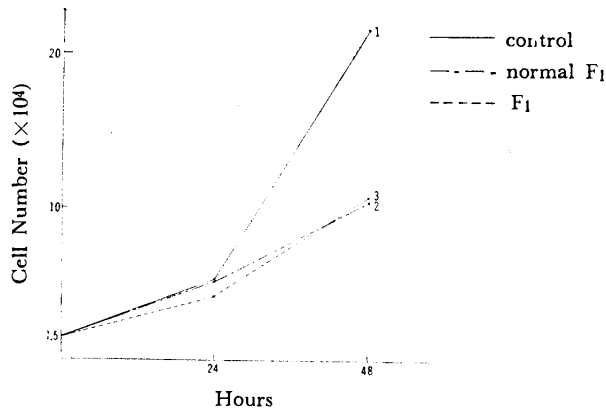    4)  mixed with lymphoid cells treated with 0.2 % trypsin
    5)  1.0 % trypsin

Fig. 3  Inhibitory effect of $F_1$ fraction. (700 ×g sediment by Schneider's
method) of sensitized lymph node cells on the proliferation of JTC-11 cells
    1)  Control. Only JTC-11 Cells
    2)  with $F_1$ Fraction of normal lymph node cells.
    3)  with $F_1$ Fraction of sensitized lymph node cells

cells.

## DISCUSSION

    In the tissue culture where the direct contact of the culture cells with
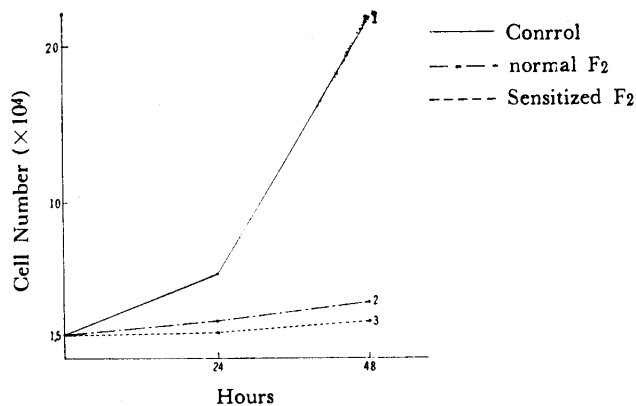sensitized lymph node cells is avoided, the question whether the target

Fig. 4   Inhibitory effect of F₂ fraction (8500×g sediment by Schneider's method) of sensitized lymphoid cell on the proliferaton of JTC-11 cells
1) Control; only JTC-11 cells
2) with F₂ fraction of normal lymph node cells
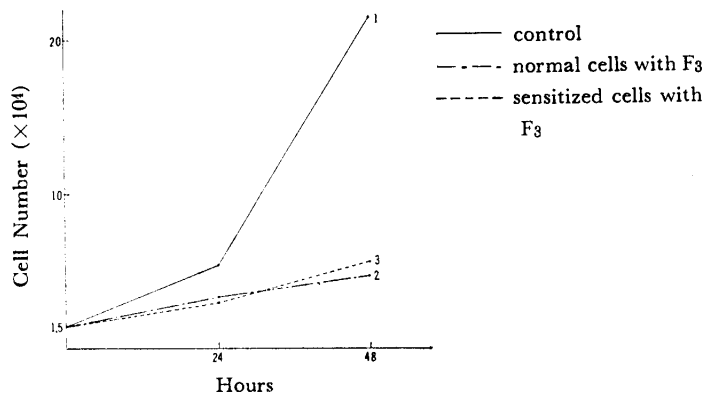3) lwith F₂ fraction of sensitized lymph node cells

Fig. 5   Inhibitory effect of F(3) fraction (100.000×g sediment by Schneider's method) of sensitized lymph node cells on the proliferation of JTC-11 cells
1) control; only JTC-11 cells
2) with F₃ fraction of normal lymph node cells
3) with F₃ fraction of sensitized lymph node cells

cells would receive any damage or not is an important problem in relation to the nature of cell antibody.   There are considerable numbers of reports (6, 7, 8) that, when the target cells and sensitized lymph node cells are cultured without direct contact, there can be observed no cell damage, but there is only one *in vitro* report by HARA (9) that cell damage is observable.   There is only one *in vivo* experiment by NAJARIAN and FELDMAN in that, when the diffusion chamber containing sensitized animal lymphoid
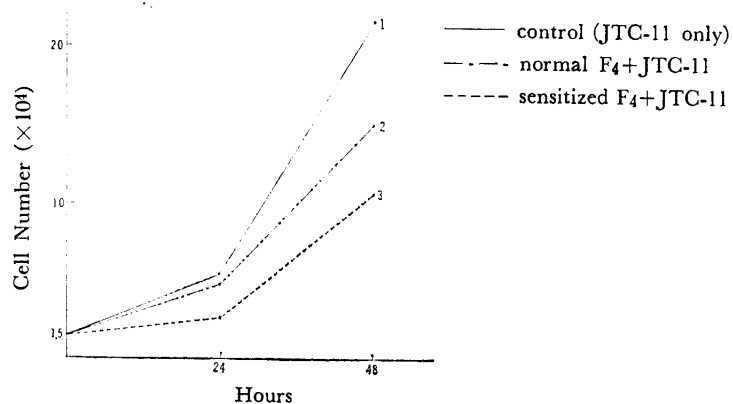
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Fig. 6 Inhibitory effect of $F_4$ fraction (100.000×g supernatant by Schneider's method) of Sensitized lymph node cells on the proliferation of JTC-11 cells
1) control: only JTC-11 cells
2) with $F_4$ fraction of normal lymph node cells
3) with $F_4$ fraction of sensitized lymph node cells

cells was placed in the peritoneal cavity or under the skin near the homografted skin, using mice or guinea pigs, the rejection of the skin graft was accelerated. In the case of HARA's experiment it is assumed that, when the number of sensitized lymph node cells in the diffusion chamber is small, there can be observed no antitumor activity at all, and the diffusible agent that passes through the Millipore filter would be very weak or quantitatively negligible.

In view of the fact that the antitumor activity is greater when sensitized lymphoid cells are in direct contact with the target cells, the diffusible agent is arbitarily assumed to be located on the cell surface of the sensitized lymphoid cells, and the trypsin treatment was carried out on this assumption. As has already been mentioned in the section of methods, more than 90 % of the sensitized lymph node cells are still alive even after the treatment with trypsin in the concentrations of 1.0 % to 0.01 %. DAVID et al. (10) likewise reported that the sensitized cells treated with 1 to 10 mg/ml trypsin lost their action against specific antigen but they still survived. They consider that trypsin deprives the sensitized cells of their specific activity because trypsin digests protein on the surface of the sensitized cells. Further, they state that such sensitized cells, when treated with RNase or DNase, do not lose their specific activity.

Concerning the localization of the diffusible agent that exhibits antitumor effect, KERN (11) in 1959 demonstrated that such antibody activity is located in the microsomes of sensitized guinea-pig lymphocytes. By

differential centrifugation WILSON and CROSBY (12) fractionated homogenates of lymph node tissues of the mice previously immunized by homotransplantation of spleen or skin into 4 fractions and examined the cytotoxic activity of each fraction on donor mouse lymphocytes by the rate of stainability with Eosin stain. As a result they found that $F_1$ fraction, containing nuclear material, debris, cell wall, and mitochondria, isolated by the centrifugation, and $F_2$ fraction, the supernatant after ultracentrifugation, show far more marked cytotoxic activity than $F_3$, microsomal fraction, and $F_4$, ribosomal fraction.

In the present experiment the antitumor activity of the regional lymph node cells from the mouse transplanted with Ehrlich ascites tumor cells has been found in the sediment ($F_2$ fraction obtained at 8,500 g for 15 min and in the supernatant ($F_4$ fraction at 100,000 g for 60 min). The precise nature remains unknown because no electron microscopic observations were made with each fraction. Nevertheless, the results of the present experiment seem to agree with the findings of WILSON and CROSBY because $F_2$ fraction corresponds to the mitochondrial fraction, and $F_4$ fraction to a soluble fraction containing a considerable amount of proteins and a portion of cell membrane by the original method of SCHNEIDER. While there are some technical differences between our procedures and those employed by WILSON and CROSBY, in that they used phosphate buffer in isolating subcellular fractions and deoxyribonuclease for adjusting homogenates but we used Hanks solution that has the least cytotoxic
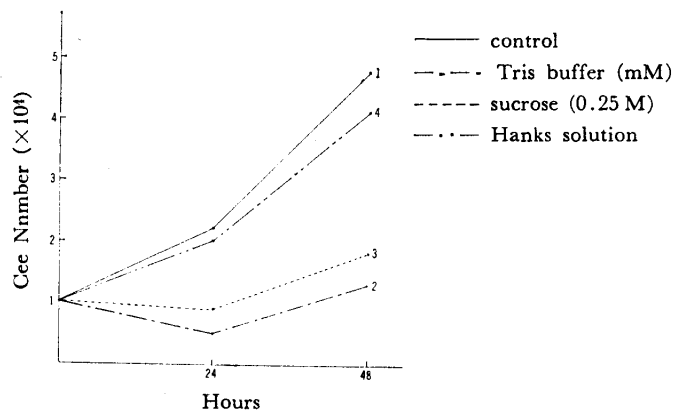


Fig. 7   Effects of the culture meclium on JTC-11 cells
1)   control: only JTC-11 cells
2)   mediunm: Tris buffer (mM)
3)   medium: Suerose (0.25 M)
4)   modium: Hanks solution

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effect on JTC-11 cells out of the three media; Hanks solution, 0.25 M sucrose and 0.01 M Tris buffer, the composition of each fraction would be essentially identical.

Hanaoka (13) extracted the membrane fraction of lymphocytes and demonstrated the antitumor activity against target cells (SCL cells) in the membrane fraction by the tissue culture method with addition of a complement. The membrane fraction was extracted by Neville's method from the sensitized lymphocytes of A/jax mouse transplanted with SCL cells. For the purposes to elucidate the localization of transplantation antigen in human leucocytes. Rapaport *et al.* (14) fractionated the human leucocytes into four fractions by the differential centrifugation technique of Schneider and further separated the supernatant fraction of 105,000 g by centrifugation at 198,000 g into supernatant and sediment fractions. As a result they found that all the fractions other than the supernatant fraction of 198,000 g contain antigenic activity. Since election microscopically all the other fractions contain the membrane component in common, it is the membrane component that has the transplantation antigen.

In view of such available findings we are of the opinion that the substance with antitumor activity is contained in the membrane component most likely present in all the four fractions separated in our experiment, because $F_2$ and $F_4$ fractions containing membrane component showed very strong antitumor activity, while $F_1$ and $F_3$ with less membrane component showing some such activity. The question whether or not the subcellular factor with antitumor activity, the factor on the surface of sensitized lymph node cell, which loses its antitumor activity by trypsin treatment, and the factor from sensitized lymph node cell that passes through the diffusion chamber are all one and the same substance has to await further study.

SUMMARY

The regional lymph node cells of the mice sensitized with Ehrlich ascites tumor cells is known to possess a substance that shows antitumor activity on target cells (JTC-11 cells). For the purpose to clarify the localization of this substance the regional lymph node cells from such sensitized mice were treated with trypsin solution of different concentrations (1.0 %, 0.2 %, and 0.01 %), and the tissue culture was carried out with JTC-11 cells. As a result it was found that these lymph node cells lost antitumor activity.

Next, by the differential contrifugation of these sensitized lymphocytes

we obtained $F_1$ fraction (700 g, sediment), $F_2$ (8,500 g sediment), $F_3$ (100,000 g sediment) and $F_4$ (100,000 g supernatant). In the presence of each of these fractions tissue culture was conducted with JTC-11 cells as target cells, and it was found that the substance with antitumor activity is contained abundantly in $F_2$ fraction (8,500 g sediment) and $F_4$ fraction (100,000 g supernatant).

After giving due consideration to the results of these two experiments and also to the available data in the literature, we assume that the substance with antitumor activity is contained in the cell membrane component.

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