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Antitumor factors of regional lymph node cells in the transplantation of Ehrlich ascites tumor cells. II. Properties of antitumor factors

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Abstract

With the purpose to elucidate further the properties of the supernatant F4 obtained by centrifugation at 100, 000 g from the regional lymph node cells of the Cb mice sensitized with EHRLICH ascites tumor cells, the supernatant (cf. Report 13) was subjected to the following treatments:. The supernatant (F4) was first diluted variously with Hanks solution. 2. F4 was passed through Seitz filter. 3. Heated at 56°C for 30 minutes. 4. It was frozen and thawed. 5. Treated with O. 01 96 trypsin solution. Each of F4 frations so treated was used in the tissue culture of JTC-II cells (derived from EHRLICH cancer cells) as target cells. As a result we found that the antitumor factor passes th rough Seitz filter, and it loses its antitumor activity by 4-fold dilution or over. Likewise F4 loses its activity by freezing-thawing treatment as well as by trypsin treatment, while by heat treatment at 56°C for 30 minutes, it still retains its activity. From these finding, it is assumed that the antitumor factor contained in F4, fraction is not serum antibody but is a protein associated with the cell membrane.

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ANTITUMOR FACTORS OF REGIONAL LYMPH NODE CELLS IN THE TRANSPLANTATION OF EHRLICH ASCITES TUMOR CELLS
II. PROPERTIES OF ANTITUMOR FACTORS

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As described in the previous paper (13), the subcellular fraction isolated from the regional lymph node cells of the mouse sensitized with Ehrlich ascites tumor cells has antitumor effect on the proliferation of the tumor cells. Further, it has been confirmed that such sensitized lymph node cells lose the antitumor activity after being treated with trypsin. From these two facts it is deduced that the antitumor factor of the sensitized lymph node cells is the immuno-protein adhered to the surface of the cell membrane.

For the purpose to elucidate the property of this antitumor factor, biological and physical properties of this substance were studied in the present experiment. As already been explained in the previous report, the supernatant fraction (F_4, at 100,000 g) shows a more marked antitumor activity and is easier to handle than the sediment fraction (F_2, at 8,500 g), hence this supernatant fraction was used in the present experiment.

MATERIALS AND METHODS

Animals: Just as in the previous experiment (13), Cb mice weighing around 20 g were employed.

Tumor cells: The cells used for transplantation were Ehrlich ascites tumor cells. These were the tumor cells maintained at the Department of Pathology, Okayama University by passing successively through the peritoneal cavity of Cb mice.

Culture cells: The culture cells used were Ehrlich ascites tumor cells (registered as JTC-11 strain).

Sensitization: Ehrlich ascites tumor cells (5×10^6 JTC-11 cells) were transplanted subcutaneously on the back between the scapulas.

Fractionation method: Two weeks after tumor transplantation, lymph nodes were removed from both sides of the neck and axilla of the mice, washed in cold Hanks solution, subjected to homogenization in a glass homogenizer by 5 rapid

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strokes of revolution and further subjected to 7 strokes of homogenization in a homogenizer of Potter-Elevehjein type while revolving the pestle with teflon head at a high speed to obtain homogenate. The medium in this instance was Hanks solution. To obtain the supernatant fraction, these homogenates were centrifuged at 100, 000g for 60 minutes. The supernatant thus obtained is taken as the antitumor factor of the sensitized lymph node cells and is used in the following experiments. This supernatant corresponds to F_4 fraction and has antitumor activity on JTC-11 cells as mentioned in the previous report.

Tissue culture: Of 10 ml supernatant treated in various ways (as will be later explained), 2 ml of it is used for the tissue culture. Two ml supernatant, 6ml YLE solution and 2 ml bovine serum are mixed and to this mixture, 10, 000—20, 000 cells/ml of JTC-11 cells are added and 1.5 ml of the mixture is placed separately in test tubes at the angle of 5°, and the stationary culture is conducted for 24 and 48 hours at 37°C.

Cell counting: By decanting the short test tubes gently the medium is discarded after 24- and 48-hour culture, 1.5 ml of crystal violet solution is then poured into the test tube to stain the culture cells attached on the vessel wall, and the cell counts are taken with Bürker-Türk hemocytometer, taking the average of three test tubes each time.

Treatment of the supernatant (F_4): For the purpose to find out the properties of the F_4 fraction, it is treated in various ways as follows:

1. *Multiple dilutions:* Two ml of supernatant is diluted with Hanks solution by the two-fold dilution method. Supernatant used was prepared in 9 ml volume from 1.02g lymph node (taken from 13 mice), and by the micro-Kyeldahl method the supernatant proved to contain 3.312 mg/ml of protein. By adding 2 ml of each dilution to the medium the tissue culture is conducted. In each tube, 10,000 cells/ml are added.

2. *Passing the supernatant through Seitz filter:* From the 15 sensitized mice 18 ml of the supernatant is prepared by the method described in the foregoing. Of this 18 ml supernatant, 16 ml is passed through Seitz filter asceptically, and the filtered and unfiltered supernatants (2 ml) are separately used in tissue culture for the comparison of their results.

3. *Heat treatment of the supernatant:* The sensitized supernatant is asceptically incubated in a hot water bath of 56°C for 30 minutes while stirring. Then 2 ml of this heat-treated supernatant is mixed with JTC-11 cells and cultured.

4. *Freezing-thawing of the supernatant:* The sensitized supernatant is left standing at the temperature of −20°C for one hour to freeze it, and is thawed in a hot water bath of 37°C to be used for the JTC-11 cell mixed culture.

5. *Trypsin treatment of the supernatant:* To 2 ml of the sensitized supernatant is added 2 ml of 0.01 % trypsin GKN solution, and after leaving this mixture in a warm room for one hour, the tissue culture with JTC-11 cells is carried out.

6. *Control groups:* To five test groups mentioned so far, 2 ml of the supernatant obtained from normal mouse lymph node cells are mixed with JTC-11 cells and cultured as the controls.

RESULTS

1. *Multiple dilution of the sensitized supernatant* (F_4) : As shown in Fig. 1, the sensitized supernatant exhibits antitumor activity up to the two-fold dilution, but from the 4-fold dilution on, there is no significant difference from the control, showing no distinct antitumor activity.

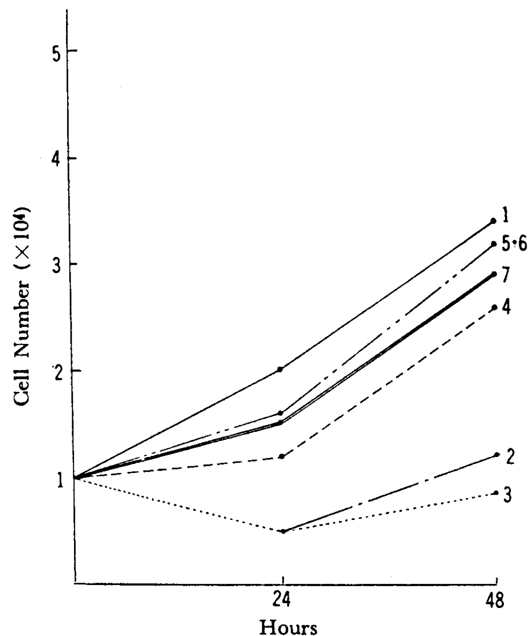

Fig. 1. Multiple dilution of supernatant
1) Control, only JTC-11 cells. 2) Supernatant (100,000×g) of sensitized lymphnode cells 3) Two-fold dilution. 4). 4-fold dilution. 5) 8-fold dilution. 6) 16-fold dilution. 7) 32-fold dilution.

2. *Filtration :* The supernatant passed through the filter has the inhibitory effect on the proliferation of JTC-11 cells just as the sensitized supernatant, showing a distinct difference from the control group. Namely, the antitumor factor in the supernatant has passed through Seitz filter. (Fig. 2).

3. As the heat treatment, freezing-thawing and the trypsin treatment have been done simultaneously in the same experiment, the results are summarized in Fig. 3. As is obvious from the figure, the supernatant heated at 56°C for 30 minutes still has the antitumor activity and inhibits the proliferation of JTC-11 cells. In contrast, the supernatant frozen at −20°C and thawed, has lost its antitumor activity, and likewise the one

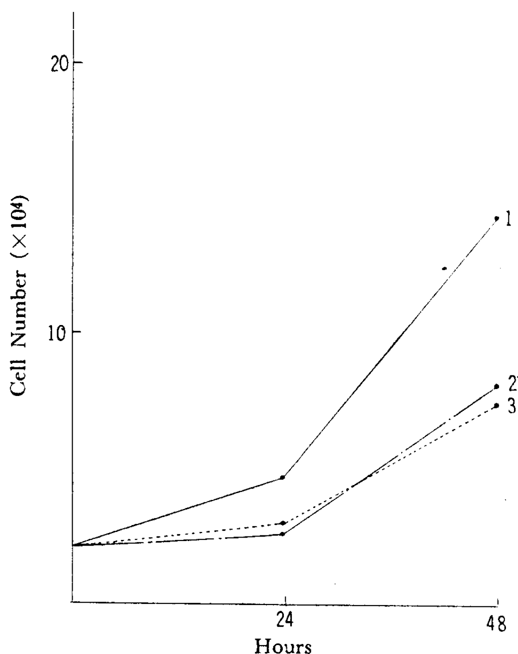

Fig. 2. Pathway taken by the supernatant of sensitized lymph node cells through the Seitz filter apparatus
1) Control, only JTC-11 cells. 2) The supernatant that passed through Seitz filter. 3) Addition of untreated supernatant.

treated with 0.01 % tyrpsin-GKN solution has lost its antitumor activity, both differing not significantly from the control groups.

DISCUSSION

As has been demonstrated in the present experiments, since the antitumor factor passes through the Seitz filter and it loses its antumor activity when treated with trypsin, this factor seems to be closely associated with protein. Furthermore, as the supernatant does not lose its activity even after heat treatment at 56°C for 30 minutes, and no complement has been added in the present experiment, the antitumor factor contained in the supernatant seems to differ from cytotoxic antibody dependent upon complement that shows cell lysis.

HANAOKA (1, 2) studied cytotoxic effect of the supernatant fraction (100, 000 g, 120 min) prepared from the membrane component of the lymphoid cells from A/Jax mice sensitized with SCL cells and treated with sodium deoxycholate and supersonicated. He determined the cyto-

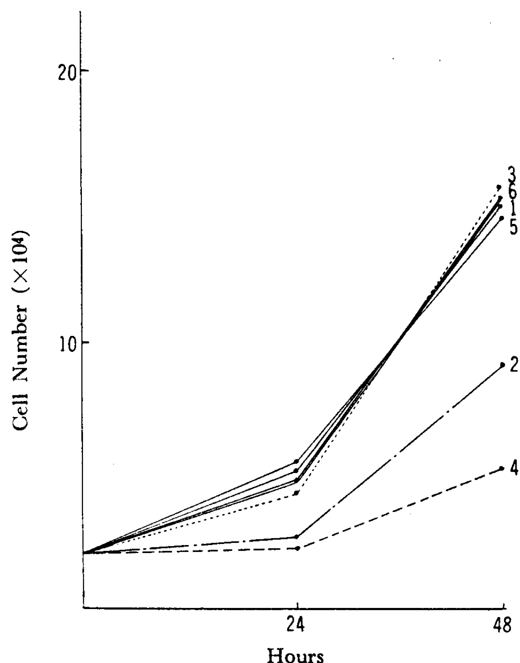

Fig. 3. Antitumor effect of treated supernatant
1) Control, only JTC-11 cells. 2) Addition of the sensitized supernatant. 3) Addition of the supernatant from normal lymph node cells. 4) Addition of the supernatant previously heated at 56°C for 30 minutes. 5) Addition of the supernatant previous frozen at −20°C. 6) Addition of trypsin-treated supernatant.

toxic activity by counting the number of surviving SCL cells after incubating SCL cells suspended in the medium of the supernatant plus complement for 2 hours at 37°C. He claims that without complement such cytotoxic activity is lost. Therefore, the antitumor factor as observed in the present experiments distinctly differs from that of HANAOKA. The cytotoxin contained in the membrane component (as in the case of HANAOKA) is in 19S fraction and is quite different from 7S globulin normally found in serum antibody. HANAOKA used as his starting material the membrane component of the lymph node cells super-immunized with lymphoid leukemic cells, whereas in our experiment we used as the starting material the lymph node 2 weeks after the transplantation of Ehrlich ascites tumor cells of epitheloid nature, sensitized relatively insufficiently. For this reason there can be seen some difference from the results of HANAOKA. However, in view of the fact that the antitumor factor loses its activity by trypsin treatment, that it passes through the diffusion

chamber, and that, when EHRLICH ascites tumor cells are cultured with regional lymph node cells, these lymph node cells aggregate onto the surface of EHRLICH ascites tumor cells leading the latter to destruction, the antitumor factor obtained in the present experiment seems to be also bound to the membrane component.

According to HARA (11), he has observed antitumor substance also in the serum of the mouse at the time of transplantation of EHRLICH ascites tumor cells. There is a possibility that such a substance in serum, being attached on the surface of lymph node cells, acts as a cellular antibody, the so-called cytophilic antibody (3, 4). GOVAERT (1960) (5) reported that by the action of humoral antibody normal cells acquire cytotoxicity. However, in view of the fact that in the incubation of normal mouse lymph node cells with the serum obtained 10 days after the transplantation of EHRLICH ascites tumor cells from the sensitizied mouse, the lymph node cells did not acquire antitumor activity, and that no cytotoxic antibody could be demonstrated in lymph node cells as reported by PERLMAN and BROBERGER (6) (1963), GRANGER and WEISSER (1964) (7), MÖLLER (1965) (8) in their precise experiments, the antitumor factor of regional axillary lymph node cells obtained after EHRLICH tumor transplantation seems not to be the antibody of circulating blood, that has become attached onto the cell surface. AKIYAMA (9) studied the appeararance of antibody in his heterotransplantation of rat ascites hepatoma cells to mice and he noted that IgM of 19S of a substance similar to it is attached onto lymphoid cells, but no IgM that has antitumor activity is found in serum. This may be due to the heterotransplnatation where gene differs markedly.

NAJARIAN and FELDMAN (1963) (10) isolated lymphoid cells from the spleen and lymph nodes of CBA mice sensitized with skin graft of A mouse, and prepared the supernatant fraction from these cells by supersonication and centrifugation at 3, 000 rpm. They demonstrased that it is possible to accomplish passive transfer of immunity with the use of such a supernatant. They further state that the soluble component of the serum is resistant to 0.1M 2-mercaptoethanol and has the slow sedimentation coefficient of about 6, resembling the so-called 7S γ-globulin. However, they have not clarified what effect this supernatant would have on the target cell *in vitro*. It is interesting to note that antibody-like substance concerned with transplantation immunity has been extracted from sensitized lymphoid cells and that such a substance resembles globulin. It is not possible to draw any definitive conclusion whether the antitumor factor that is liberated from the regional lymph node cells and after passing through the diffusion chamber, acts inhibitorily on the growth of EHRLICH

tumor cells as suggested by HARA in 1965 (11) is identically the same substance as the antitumor factor contained in the supernatant of the subcellular component of lymph node cells of the present experiment. However, from the fact that either one of such a factor wields antitumor activity without complement and that both viable lymph node cells and the supernatant are inactivated by trypsin treatment, and from the data presented by various workers, the substance that shows an inhibitory effect on JTC-11 cells seems to be a protein that can be liberated to some extent from the cell membrane of lymph node cells, and such a substance will be a component of the cell membrane or a substance attached to the membrane. Further, as reported by MANNAMI (1968) (12) the fact that the sensitized lymph node cells *in vitro* aggregated on JTC-11 cells, fused with each other and both cells were destroyed seems to suggest the presence of antitumor substance in the cell membrane of sensitized lymph node cells.

SUMMARY

With the purpose to elucidate further the properties of the supernatant F_4 obtained by centrifugation at 100, 000 g from the regional lymph node cells of the Cb mice sensitized with EHRLICH ascites tumor cells, the supernatant (cf. Report 13) was subjected to the following treatments:

1. The supernatant (F_4) was first diluted variously with Hanks solution. 2. F_4 was passed through Seitz filter. 3. Heated at 56°C for 30 minutes. 4. It was frozen and thawed. 5. Treated with 0.01 % trypsin solution. Each of F_4 frations so treated was used in the tissue culture of JTC-11 cells (derived from EHRLICH cancer cells) as target cells. As a result we found that the antitumor factor passes th rough Seitz filter, and it loses its antitumor activity by 4-fold dilution or over. Likewise F_4 loses its activity by freezing-thawing treatment as well as by trypsin treatment, while by heat treatment at 56°C for 30 minutes, it still retains its activity. From these finding, it is assumed that the antitumor factor contained in F_4 fraction is not serum antibody but is a protein associated with the cell membrane.

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