Antitumor factor of regional lymph node cells in the transplantation of Ehrlich tumor cells. 3. Passive transfer of the antitumor factor

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Antitumor factor of regional lymph node cells in the transplantation of Ehrlich tumor cells. 3. Passive transfer of the antitumor factor∗

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Abstract

In vitro and in vivo experiments were conducted for the purpose to determine whether or not the antitumor factor found in the regional lymph node cells of the mouse sensitized with Ehrlich tumor cells would transfer its antitumor activity to normal lymph node cells. In in vivo experiments normal lymph node cells incubated at 5°C for 60 minutes in the supernatant containing the antitumor activity have shown the antitumor activity against JTC-11 cells in mixed culture. Namely, it has been demonstrated that the antitumor activity in the supernatant can be transferred directly to normal lymph node cells in vitro. In the in vitro experiments, the same results as in in vivo experiments were obtained. The antitumor activity against JTC-11 cells has been detected in the lymph node cells obtained on 4, 7 and 9 days after subcutaneous and intraperitoneal injections of the supernatant having antitumor activity. Next, we tried DNase and RNase treatments of the sensitized supernatant to observe the transfer factor-like substance. The results indicate that, while the passive transfer is possible with the supernatant treated with DNase, it is not with the RNase-treated supernatant. From these findings it is assumed that the factor (in the sensitized supernatant) capable of conferring the antitumor activity is an RNA-dependent substance (or a substance closely associated with RNA) and is probably different from the antitumor factor reported in Parts 1 and 2.

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In our previous report we have already demonstrated that the subcellular fraction (100,000 g supernatant) of the regional lymph node cells from the mouse sensitized with Ehrlich ascites tumor cells contains an antibody-like substance different from the so-called humoral antibody. According to Najarian and Feldman (1), it is possible to accomplish "passive transfer" of transplantation immunity in vivo and in vitro with γ-globulin-like substance extracted from the sensitized lymph node cells at the time of homotransplantation. In the present experiments we investigated to see whether or not the subcellular factor (100,000 g supernatant) extracted from the regional lymph node cells after the transplantation of Ehrlich cancer cells to mice would enable us to accomplish passive transfer in vitro and in vivo. As a result we found that such a supernatant contains an RNA-dependent substance probably different from the antibody-like substance that shows a direct antitumor activity, suggesting that this factor acts like the transfer factor. The present report describes the results obtained in this investigation.

MATERIALS AND METHODS

Animals: Just as in Part 1. Cb mice of inbred strain, two months old weighing about 21 g were used. These mice are bred and maintained at the Okayama University Mouse Colony.

Tumor cells: The cells used were Ehrlich ascites tumor cells just as in Parts 1 and 2.

Culture cells: The cells were JTC-11 cells (derived from Ehrlich cancer cells) and these cells are known to form tumors in mice.

Sensitization: Tumor cells (5 × 10⁶ cells/ml) were transplanted subcutaneously on the back between scapulas of Cb mice, 14 days after the transplantation regional lymph nodes (from neck and axilla) were taken out, cut into small pieces, washed with Hanks solution, homogenized, and the cell suspensions
so prepared were used for the experiments.

**Antitumor factor:** As the antitumor factor the supernatant fraction, $F_4$, was employed as in Part 1. This is prepared with the regional lymph node cells of Cb mouse. Namely, these lymph node cells were homogenized in the glass homogenizer of Potter-Elevehjem, the homogenates were subjected to ultracentrifugation (Hitachi Ultracentrifuge) at 100,000 $g$ for 60 minutes and the supernatant thus obtained served as $F_4$. This supernatant (as already reported in Part 1) has antitumor activity on JTC-11 cells.

**Experimental**

1) **In vitro passive transfer**

Twelve Cb mice are sensitized with Ehrlich ascites tumor cells, 14 days later the regional lymph nodes are taken out and homogenates are prepared as mentioned in the foregoing, and the homogenates are centrifuged at 100,000 $g$ to obtain the supernatant fraction. This fraction is kept at 5°C to be used for the experiments.

Next, from 6 normal Cb mice both axillary and cervical lymph nodes are removed, cut into small pieces in cold Hanks solution, these pieces are passed through 150-mesh filter, washed well to remove serum, kept at 5°C to be used as the control normal lymph node cells. By Eosin-Y staining it has been proven that about 90% of these cells are alive.

To 3 ml of the supernatant prepared as described above (corresponding to 3 sensitized mice) is added $7.5 \times 10^5$ cells/ml of normal lymph node cells and the mixture is incubated at 5°C for 60 minutes, while shaking the test tubes every ten minutes. After incubation, the material is centrifuged at 1,5000 rpm, by discarding the supernatant, the sediment is washed with Hanks solution 3 times, and these cells are cultured with JTC-11 cells.

2) **In vivo passive transfer**

The animals, tumor cells and culture medium used are the same as in the in vitro experiments. For the antitumor factor, 100,000 g supernatant of the sensitized mouse regional lymph node cells is used.

To 6 Cb mice 1.5 ml each of the sensitized supernatant is injected subcutaneously on the back and intraperitoneally to the total of 3 ml (equivalent to 3 sensitized mice). Four, 7 and 9 days after the injection, 2 mice each are sacrificed, the regional lymph nodes are taken out, these are cut into small pieces, washed, passed through the 150-mesh filter, and the filtrates so prepared served as "supernatant-treated lymph node cells". With these lymph node cells and JTC-11 cells the mixed tissue culture is carried out.

3) **In vivo passive transfer by the supernatant treated with DNase or RNase**

By mixing the sensitized supernatant with 0.1 mg/ml DNase and incubating the mixture at 37°C for two hours, 1.5 ml each of the incubated mixture is injected subcutaneously on the back and intraperitoneally to normal Cb mice. Nine days after the injection, regional lymph nodes are removed from the animals,
and these lymph nodes are treated in the same manner as with the in vivo passive transfer and the cell suspension is prepared with Hanks solution, and this cell suspension is mixed with JTC-11 cells and cultured for 24 or 48 hours at 37°C. In the case of RNase treatment the identical procedures are carried out. The control groups for these experiments are prepared in exactly the same manner as in the in vivo passive transfer.

Tissue culture: As in Part 1 (I) JTC-11 cells are used as the culture cells.

1) In vitro passive transfer

To 10 ml medium 20,000 cells/ml JTC-11 cells and 7.5 × 10⁶ cells/ml of the sensitized lymph node cells are added, and 1.5 ml each of the mixture is put into individual test tubes, lanted at an angle of 5° and incubated at 37°C for 24 or 48 hours. The medium used is consisted of the mixture of YLE solution and inactivated bovine serum (8:2, v/v).

2) In vivo passive transfer

The mixture of 20,000 cells/ml of JTC-11 cells and 2×10⁶ cells/ml of treated lymph node cells is put into 10 ml medium. Next, 1.5 ml each of this mixture is placed in short test tubes, and the stationary culture is conducted at 37°C for 24 or 48 hours.

3) In vivo passive transfer with the supernatant treated with DNase or RNase

Culture methods employed in this experiment are identical with those used in the in vitro passive transfer.

RESULTS

The in vitro passive transfer, in contrast to that of the controls, shows the inhibitory effect on the growth of culture cells within 24-hour culture as shown in Fig. 1, and by 48-hour culture it reveals a marked difference from the control. It is obvious that the sensitized supernatant contained a substance capable of transferring the antitumor activity in vitro to normal lymph node cells.

In the case of in vivo passive transfer, the regional mouse lymph node cells 4 days after the injection of the supernatant show no difference from the control. With those lymph node cells obtained 7 days after the supernatant shown in Fig. 1. In vitro Passive Transfer 1) control, only JTC-11. 2) Effect of normal lymph node cells treated with supernatant without antitumor activity on JTC-11 cells. 3) Effect of normal lymph node cells after treatment with the supernatant having antitumor activity on JTC-11 cells.
injection, the sensitized group shows clearly the growth-inhibitory activity by 24-hour culture, but by 48-hour culture there is no difference from the control as shown in Fig. 2. With those lymph node cells obtained 9 days after the injection, although culture conditions are not so good, both at 24- and 48-hour cultures they exhibit some growth inhibitory effect as compared with the controls (Fig. 2), proving that passive transfer of anti-tumor activity is accomplished.

As for passive transfer by the DNase treatment of the supernatant (Fig. 3), the antitumor activity both at the 24-hour and 48-hour cultures differs significantly from that of the controls, indicating that DNase has
had not any effect on a transfer factor-like substance in the sensitized supernatant. On the other hand, in the case where the supernatant is treated with RNase, at 24-hour culture there is no difference from the controls as illustrated in Fig. 4, whereas at 48-hour culture the sensitized group, on the contrary, shows a slight proliferation as compared with the normal control. It is evident that RNase acts on the transfer-like substance of the sensitized supernatant as to nullify its transfer capacity.

**DISCUSSION**

As so far described, we have demonstrated that both *in vitro* and *in vivo* the subcellular fraction (100,000 g supernatant) of the regional lymph node cells from the mouse transplanted with Ehrlich cancer cells contains a substance that might be called "transfer factor" of Lawrence (8). Now, there arises a question what this substance really would be. When this supernatant is mixed with normal lymph node cells and incubated, there appears antitumor activity in the cells or when the supernatant is first injected into the mouse, and the regional lymph node cells taken at certain time intervals from the injected mice are made to act on JTC-11 cells, there can also be seen growth-inhibition of JTC-11 cells.

Recently, it is being clarified that RNA and ribosomes play an important role as the mediator in immune reaction. In the field of transplantation immunity likewise Mannick and EgdaHL (2) have observed transfer reaction with RNA treatment. That is, when RNA extracted from the regional lymph node cells of the rabbit B transplanted with rabbit C skin is incubated with the lymph node cells of the another rabbit A at 37°C for 15 minutes, then the RNA-treated lymph node cells are injected subcutaneously into the rabbit C, 2 to 3 days later there develops reddish swelling. This result indicates that the extracted RNA contains the transfer factor-like substance. Later Mannick (3) has demonstrated that transfer immunity could be conferred to the normal rabbits previously injected with sensitized lymph node whole cells. By the homograft experiments with mice Gerughty and coworkers (4) have observed *in vitro* transfer immunity of ribosomes. Namely, when the ribosomes from the spleen cells of the BALB/c mouse transplanted with L cells (derived from C3H mouse) are incubated with normal BALB/c mouse spleen cells, these normal spleen cells acquire cytotoxic activity to destroy L cells in mixed culture. When ribosomes are previously treated with RNase, it is said that transfer activity can be eliminated. Similar findings have been observed *in vitro* with rats by Wilson and Wecker (5). In the field of microbiology and virology...
also there are reports stating that RNA-dependent antigen complex of phagocytes can be transferred to lymphocytes and these lymphocytes acquire immunity.

The transfer factor-like substance in the 100,000 g supernatant in this experiment is inactivated by RNase but not by DNase. Therefore, this substance seems to be an RNA-dependent factor.

On the other hand, LAWRENCE (7, 8) has obtained an extract from the leukocytes of a person showing delayed hypersensitivity, which imparts the same type of hypersensitivity when it is injected to another person not showing hypersensitivity, and he calls the active component of the extract as "transfer factor". This transfer factor is not desensitized by DNase or RNase nor by DNase plus trypsin treatment, and its real nature seems to be still obscure. Powell and coworkers (9) have also found a transfer factor-like substance. They obtained regional lymphoid cells 11 to 50 days after the transplantation of guinea pig skin homografts, subjected these lymphoid cells to freezing-thawing repeatedly 10 times, then centrifuged at 105,000g, the 105,000g supernatant thus obtained was dialyzed against distilled water, and they found the sediment to contain a transfer factor. This transfer factor-like substance has very interesting properties in that it is not at all affected by DNase while its activity is rather increased by RNase, and it is inactivated by trypsin. They consider arbitrarily the essential component of this transfer factor-like substance to be protein. This substance resembles quite closely the transfer factor of Lawrence but it differs in that it loses its activity when treated with trypsin. In a series of experiments with mice, Najarian and Feldman (1) have noted a transfer factor in sensitized lymphoid cells, which they consider to be quite similar to γ-globulin.

These transfer factor-like substances so far mentioned do not have a uniform common property but these differences can accounted for the differences in the species of animals and the methods of fractionations employed. It remains a problem whether or not the transfer factor observed by Lawrence, Powell, and Najarian-Feldman would transfer immunity in vivo or in vitro. Moreover, this transfer factor mentioned by these three groups of workers seems to be a protein-dependent substance and not an RNA-dependent one. However, the transfer factor as proposed by Mannick and Godahl, Gerughty and coworkers, Wilson and Wecker, including that observed in our experiments, seems to be an RNA-dependent substance both in vitro and in vivo.

The question from what cell in regional lymph node the transfer factor is derived and what is the pathway by which normal lymph node
acquire immunity remains a problem to be solved in future. From the fact that, although the trypsin-treated cells lose the direct activity on antigens, they retain the potency for adoptive immunity as reported by DAVID and coworkers (10) and others, the regional lymph node cells of the mouse transplanted with Ehrlich cancer cells in the present experiment seems to contain two kinds of substances; the one is a protein-dependent cytophilic antibody-like substance that is inactivated by trypsin but wields antitumor effect directly on target cells (11) and the other, which probably has no direct effect, is an RNA-dependent substance that transforms normal lymphoid cells to sensitized lymphoid cells.

SUMMARY

In vitro and in vivo experiments were conducted for the purpose to determine whether or not the antitumor factor found in the regional lymph node cells of the mouse sensitized with Ehrlich tumor cells would transfer its antitumor activity to normal lymph node cells.

In in vivo experiments normal lymph node cells incubated at 5°C for 60 minutes in the supernatant containing the antitumor activity have shown the antitumor activity against JTC-II cells in mixed culture. Namely, it has been demonstrated that the antitumor activity in the supernatant can be transferred directly to normal lymph node cells in vitro.

In the in vitro experiments, the same results as in in vivo experiments were obtained. The antitumor activity against JTC-II cells has been detected in the lymph node cells obtained on 4, 7 and 9 days after subcutaneous and intraperitoneal injections of the supernatant having antitumor activity.

Next, we tried DNase and RNase treatments of the sensitized supernatant to observe the transfer factor-like substance. The results indicate that, while the passive transfer is possible with the supernatant treated with DNase, it is not with the RNase-treated supernatant. From these findings it is assumed that the factor (in the sensitized supernatant) capable of conferring the antitumor activity is an RNA-dependent substance (or a substance closely associated with RNA) and is probably different from the antitumor factor reported in Parts 1 and 2.

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