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Abstract

MC-induced sarcomas produced under the skin on the back between scapulas of C3H mice were transplanted successively to the mice of the same strain. Using the first and the second generation tumors, viable tumor cells were prepared and with these tumor cells C3H mice were inoculated. From these sensitized mice regional lymph nodes were taken out at certain intervals and lymph-node cells were prepared. These tumor cells were mixed with regional lymph-node cells in the ratio of 1:10, and the mixed cells were transplanted subcutaneously on the back of C3H mice, and the development and growth of tumors were observed at intervals. As a result it was found that the inhibitory effect of these regional lymph-node cells on the tumor growth was strong one to two weeks after the transplantation, but beyond 3, or 4 weeks no inhibition was observable. In connection with the present in vivo experiments, some comments were made on the available literature, and it has been demonstrated that even in the cancer-bearing animal destined to die of tumors, at certain stage of cancer there is seen an inhibitory effect of the host on the tumor growth by way of the lymphoid system and that such a response of the host seems to be correlated well with in vitro reaction.

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INHIBITORY EFFECT OF ISOLOGOUS REGIONAL LYMPHNODE CELLS FROM THE METHYLCHOLANTHRENE-INDUCED SARCOMA BEARING MICE ON THE TUMOR IN VIVO

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Reports are already available concerning the antigens that are present in tumors induced by oncogenic virus or chemical carcinogens, but they are lacking in the host (1, 2, 3). The majority of these antigens are identified by the transplantability and the manner of proliferation of viable tumor cells in the host after isotransplantation. Namely, after isotransplantation of experimentally induced tumor to superimmunize the host, the tumors grown to a certain size are either ligated or taken out, or heavily irradiated tumor cells are transplanted, and then viable tumor cells are transplanted to these animals to determine their transplantability and proliferation (3, 4, 5, 19). At the present, we have no unequivocable evidence to prove whether or not antitumor activity is at work on the tumors of the cancer bearing host in which cancer is aggravating, identically in the same manner as in the highly immunized animal. With purposes to clarify this problem, Hara (13) and Satoh (17) of our laboratory have performed transplantation experiments of Ehrlich cancer and A-cells (established mammary cancer cells) in vivo and in vitro and found that even in such mice destined to die of tumors there appears at relatively early stage of cancer, antitumor activity against its own tumors by virtue of immunological mechanism working from the lymphoid system.

However, in these studies of the isotransplanted cancer as well as homotransplanted cancer of established strain, it seems that in all probability, aside from tumor specific antigen, genetic differences would intervene strongly between the host and cancer so that it would be not sufficient to elucidate the immune reaction (antitumorigenicity) of the host against tumor antigens by their studies alone.

In view of this, we studied the correlation between the tumor proliferation and the antitumorigenicity of cancer-bearing mice. In these experiments first we produced methylcholanthrene (MC)-induced tumors by
inoculating MC, a chemical carcinogen known to induce in mice tumors with strong tumor specific antigens, and successively isotransplantations of these MC-induced tumors had been carried out into mice. Next, using the first and second transplants when tumor antigens would most likely have not undergone any alteration, the isotransplantations were conducted again. As a result we have been able to demonstrate that at a certain stage of cancer growth clearly there occurs antitumor activity in the tumor bearing host.

MATERIALS AND METHODS

**Animals**: The animals used were C3H male mice (H-2^k_), weighing about 20 g, 2 to 3 months old, and supplied from the Okayama University Mouse Colony.

**Carcinogen**: The carcinogen in the experiments was a chemical agent of aromatic carbohydrate, 20-methylcholanthrene (MC) which was suspended in arabian gum and used.

**Experimental tumors**: By the subcutaneous injection of 1 mg/mouse MC to mice on the back between scapulas MC-induced tumors were produced, and 3—4 months later when the MC-induced sarcomas grew to the size of 1 cm in diameter there were taken out and successively transplanted.

**Preparation of free tumor cells**: By a modification of Madden and Burk's method (11) the tumors of the first and second transplants in successive series are excised, cut into fine sections, and left standing in the cold 0.2 % trypsin solution mixed Hanks solution containing a few droplets of 0.04 % DNase for about two hours, while shaking occasionally. Next, after discarding the supernatant a fresh 0.2 % trypsin solution containing a few droplets of DNase was added, stirred for one hour with a magnetic stirrer, filtered through the 80-mesh filter, centrifuged at 1,000 rpm for 10 minutes, and free sarcoma cells were taken out and the numbers of nuclei were counted. The percentage of viable cells thus obtained proved to be 90—95 % by the trypan blue test.

**Preparation of lymph node cells**: Under the skin on the back between scapulas of C3H mice 500 × 10^4 cells/mouse of the first and the second transplants MC-induced tumor cells were transplanted, at certain intervals regional non-metastatic lymph nodes (superficial and deep lymph nodes of the neck, axilla and the upper arm on both sides) were excised, finely sliced, immersed in Hank's solution to have cell suspension, then centrifuged at 2,000 rpm for 5 minutes, and washed, repeating the procedures 3 times to obtain free lymph node cells to be used for the neutralization test. For the control groups, lymph nodes from the same regions of normal C3H mice were taken out and subjected to the identical treatments as with cancer-bearing mice to serve as the controls (normal lymph node cell suspensions).
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EXPERIMENTS

Exp. 1: Determination of successively transplantable sarcoma cell numbers.

Viable sarcoma cells prepared by the above-mentioned method of MADDEN and BURK were divided into five groups; $5 \times 10^4$ cells/ml, $1 \times 10^5$ cells/ml, $2 \times 10^5$ cells/ml, $4 \times 10^5$ cells/ml, and $11 \times 10^5$ cells/ml, and each milliliter of them was transplanted to C3H mice on the back under the skin between scapulas to determine successively transplantable cell numbers.

Exp. 2: Antitumorgenicity of regional lymph nodes from cancer-bearing mice

At every one, two, 3, 4 and 8 weeks after the MC-induced-sarcoma cell transplantation 3—4 mice each were sacrificed, regional lymph nodes collected, and lymph node cells were prepared to be used for the neutralization test. Namely, the test groups are divided into 5 groups of 10 mice each, and at first $2 \times 10^6$ cells/ml, the minimum, successively transplantable sarcoma cell numbers as determined in Exp. 1, is added $2 \times 10^6$ lymph node cells/ml obtained, 1, 2, 3, 4, and 8 weeks after the transplantation from respective groups of cancer-bearing mice, and the mixture is transplanted subcutaneously on the back of C3H mice of each group. Each group always has its control in which sarcoma cells alone are transplanted. As for the control groups the mixture of normal mouse lymph node cells plus sarcoma cells in the same ratios as in the test groups is transplanted subcutaneously on the back of C3H mice in respective groups. The growth of cancer is estimated periodically; namely, the time required for the tumor to grow palpable and the growth thereafter by a calibrator.

Exp. 3: Antitumor activity of regional lymph nodes from the mice removed of tumors

By transplanting $100 \times 10^4$ cells/mouse of MC sarcoma cells to C3H mouse subcutaneously on the back, about 20 days later when the tumor grew to 1 cm in diameter, it was excised. One week after the tumor removal regional lymph node cells were prepared from the same mouse and these cells were mixed with sarcoma cells in the same proportion as in Exp. 2, and transplanted on the back of C3H mouse, and observations on the growth of tumors were done in the same manner as in the preceding experiment.
RESULTS

Exp. 1  *The determination of successively transplantable sarcoma cell numbers*

The percentage of transplantability of MC-induced sarcomas of the first and the second generations is as shown in Table 1. When the viable sarcoma cells are subcutaneously transplanted in the numbers over $2 \times 10^5$ cells/mouse, in more than 90% of the mice tumor takes hold and grows, leading the mice to death from tumors.

Exp. 2  *Antitumorgenicity of regional lymph-node cells from cancer-bearing mice*

The time required for the tumors to grow palpable and the growth curves in the cases of those mice transplanted with the mixture of sarcoma cells plus lymph node cells from cancer-bearing mice, and with the mixture of sarcoma cells plus normal untreated mouse lymph node cells are shown in Figs. 1—6. Fig. 1 illustrates the data from the groups given the mixture

<table>
<thead>
<tr>
<th>Tumor cell number inoculated</th>
<th>Number percent of tumor developing animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>$5 \times 10^4$/cc</td>
<td>50% (4/8)</td>
</tr>
<tr>
<td>$1 \times 10^5$/cc</td>
<td>75% (9/12)</td>
</tr>
<tr>
<td>$2 \times 10^5$/cc</td>
<td>90% (9/10)</td>
</tr>
<tr>
<td>$4 \times 10^5$/cc</td>
<td>100% (9/9)</td>
</tr>
<tr>
<td>$11 \times 10^5$/cc</td>
<td>100% (9/9)</td>
</tr>
</tbody>
</table>

Note: In each experiment the same tumor cell suspension was used.
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of untreated normal mouse lymph node cells. In this instance just as in the group transplanted with tumor cells only, 4 mice of the five show the growth of tumors, indicating that normal mouse lymph node cells have no antitumorgenicity. Figs. 2 and 3 illustrate the results of the cases given

Fig. 2 Growth curves of the tumors after the inoculation of the MC-induced sarcoma cells with or without lymph node cells from the tumor bearing animals, one week after the tumor transplantation
Solid lines: Tumors developed from the tumor inoculant without lymph node cells (three out of four animals developed tumors)
Broken lines: Tumors developed from the tumor cells inoculated with lymph node cells from the tumor bearing animal (two out of four animals developed tumors)
+ : Death

Fig. 3 Growth curves of the tumors after the inoculation of the MC-induced sarcoma cells with or without lymph node cells from the tumor bearing animals, two weeks after the tumor transplantation
Solid lines: Tumors developed from the tumor inoculant without lymph node cells (three out of five animals developed tumors)
Broken lines: Tumors developed from the tumor cells inoculated with lymph node cells from the tumor bearing animal (one out of five animals developed tumors)
+ : Death
Fig. 4 Growth curves of the tumors after the inoculation of the MC-induced sarcoma cells with or without lymph node cells from the tumor bearing animals, three weeks after the tumor transplantation.

Solid lines: Tumors developed from the tumor inoculant without lymph node cells (three out of four animals developed tumors)

Broken lines: Tumors developed from the tumor cells inoculated with lymph node cells from the tumor bearing animal (two out of three animals developed tumors)

+ : Death

Fig. 5 Growth curves of the tumors after the inoculation of the MC-induced sarcoma cells with or without lymph node cells from the tumor bearing animals, four weeks after the tumor transplantation.

Solid lines: Tumors developed from the tumor inoculant without lymph node cells (five out of five animals developed tumors)

Broken lines: Tumors developed from the tumor cells inoculated with lymph node cells from the tumor bearing animal (five out of five animals developed tumors)

+ : Death
the mixture of lymphnode cells obtained one and two weeks after tumor transplantation. In contrast to the controls where three mice of the four and three mice of the five transplanted with sarcoma cells alone respectively show tumor growth, with corresponding test groups receiving the mixture of the first week lymph node cells only two mice of the four and only one out of the five mice given the mixture of the second week lymph node cells reveal the transplantability and tumor growth. In addition, in the group transplanted with the first week lymph node cells the time required for the tumor to be palpable and the survival time are prolonged as compared with the control. Figs. 4, 5, and 6 respectively show the results of those groups given the mixture of the third, fourth and eighth week lymph node cells, and the tumor cells. In the group given the mixture of the third week lymph node cells two mice out of three, and five out of five given the fourth week lymph node cells show the survival and the growth of tumor, which hardly differ from the controls receiving only tumor cells.

In the cases receiving the regional lymph node cells of the eighth week after transplantation plus tumor cells, two mice of the four had
tumors developed differing from the controls receiving sarcoma cells alone where 4 mice of the five had developed tumors, whereas there is no difference in the time required for the tumors to become palpable and the length of survival of the host, indicating that the regional lymph node cells of mice 8 weeks after transplantation have lost antitumor activity.

Exp. 3 Antitumor activity of regional lymph node cells from the mice removed of tumors

In the group receiving the transplantation of sarcoma cells alone 4 mice out of the five reveal the tumor transplantability, and in the group receiving the mixture of sarcoma cells plus mouse regional lymph node cells one week after the removal of tumors two mice out of the 5 show the tumor development. Further, the death from tumor takes place two and half months after the onset of tumor in these groups.

From these findings it is evident that in the regional mouse lymph-node cells of the first week and the second week mixed with tumor cells a strong antitumor activity can be observed. Such regional lymph-node cells of later than the fourth week show no antitumor activity, but in the group receiving the mixed transplantation of such regional lymph-node cells with sarcoma cells there can be seen no tendency rather to accelerate the tumor growth in the host animals. The regional lymph-node cells of the mouse extracted of its tumors exhibited a stronger antitumor activity than that of the regional lymph node cells of cancer-bearing mouse.

DISCUSSION

Up to the present most of the tumors used in immunological studies of cancer are tumors of established strain maintained successively for a long period of time, and such tumors are transplanted to animals for immunization. However, when the tumor is maintained successively for a long time, there occurs alteration in its antigenicity so that a specific tumor is converted to a non-specific one. Therefore, even when such a tumor is transplanted to the animals of oncogenic strain, an immunogenetic factor other than cancer specific antigen inevitably intervenes between the host and the cancer transplant as to make the presence of truly specific immunity of cancer dubious (1, 2, 26). Even MC-induced sarcoma said to possess specific antigen, when successively maintained for a long time, is known to lose its specific antitumor activity, as it is converted to a non-specific antigen (15). For this reason in the elucidation of tumor immunity it is necessary to use tumor at the early stage of its development.

In the isotransplantation of a tumor to a test animal a mechanism
similar to the homotransplantation immunity is offered in explaining the rejection phenomenon on the part of the host (9, 24). Antibodies can roughly be classified into humoral antibody and cellular antibody, but the susceptibility to these two antibodies differs with tumors (21). It is said that tumors like leukemia and lymphoma that have developed in the hematopoietic organs are markedly susceptible to anti-serum of the host (8), and sarcoma and cancer cells are poorly susceptible in most cases (2), and the rejection reaction of the host against sarcoma and cancer cells largely depends upon the cells of the lymphoid series (4, 10, 14, 24). It is also reported that in the isotransplantation of lymphoid cells from the superimmunized antitumorigenic mouse to normal mouse of the same strain in vivo, antitransplantability is conferred to the recipient animal (3).

KLEIN and co-workers (1960) (5) may be said to be the first who demonstrated antitumor activity (antitumorigenicity) in the lymphoid cells of the mouse transplanted with MC-induced sarcoma. YOSHIDA and SOUTHAM (1963) (7) in their neutralization test with the spleen cells prepared from the mouse bearing MC-induced sarcoma, found that the growth of tumor in the host is similarly inhibited by spleen cells. However, reports on periodical alterations in the antitumorigenicity of lymphoid cells of cancer-bearing host in vivo are very rare (3, 18, 23). OHSUGI and associates (1966) (20), using MC-sarcoma induced under the skin on the back of C3H mouse, performed isotransplantation of MC-induced sarcoma to mice of the same strain, and preparing periodically regional lymph node cell from the host by the same technique as in the present experiments conducted the mixed cultures of tumor cells with these regional lymph node cells in vitro. They found antitumor activity in these regional lymph-node cells later than 13 days after the tumor transplantation. ROSENAU and MORTON (1966) (18) produced subcutaneously MC-induced sarcomas in both hind legs of C3H and C57BL mice, and using the third to the sixth generation tumors of the successive isotransplantation they sensitized the animals. Then they found tumor-specific inhibition of growth of MC-induced sarcoma in vivo and in vitro by sensitized isologous lymphoid cells prepared from the spleen of sensitized hosts. As a result they concluded that there was a close correlation between the inhibitory effect of sensitized lymphoid cells on tumors in vivo and the suppressive effect of such lymphoid cells on these neoplasms in tissue culture. As to the studies on the tumor growth and the antitumor activity of lymphoid cells from the host there are reports by HARA and SATOH of our laboratory, using tumors other than MC-induced sarcomas. HARA (1965) (13) studied with lapse of time the antitumor activity in vitro of sensitized lymphoid cells and spleen cells

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obtained from the host sensitized with JTC-11 cells (derived from Ehrlich ascites tumor cells) and found a strong inhibitory effect in vitro on tumor cell growth by regional lymphoid cells of the host in the first and the second week after transplantation of JTC-11 cells. SATOH (1966) (17) sensitized C3H mice by inoculating subcutaneously on the back with $5 \times 10^6$ cells/mouse, using A cells derived from the same strain mammary cancer and tried the neutralization tests in vitro with regional lymph-node cells prepared periodically from the host. He found that the regional lymph-node cells from the host 10 days after transplantation showed the strongest inhibitory effect on tumor growth, but those lymph-node cells obtained from the host at the terminal stage of cancer revealed much lower inhibitory effect.

Similar to the results of these in vitro experiments, in the present study in vivo using the first and the second transplants of MC-induced sarcomas, we found that the regional lymph-node cells obtained with lapse of time from the cancer-bearing hosts at one and two weeks after transplantation showed a strong inhibitory effect on tumor growth.

From these homo- and isotransplantations of tumors it may be assumed that the host about 7 days after cancer transplantation considers cancer antigen as not-self and acts inhibitorily on the tumor growth by way of the regional lymph-node cells, but when the cancer grows to a certain size such an inhibitory effect of the host diminishes or disappears. It seems that such an decrease in the antitumor activity of the host can be recovered to some extent by removing tumors from the cancer-bearing host. Usually, the reason why the cancer-bearing animal does not show any inhibitory effect on tumor growth seems to lie in the fact that the host is immunologically tolerant to excessive cancer antigens (25) or the immune capacity of the host has been affected in some way by tumor (21, 22). Another point to be mentioned is, as demonstrated with MC-induced mouse sarcoma by BUBENIK and KOLDOVSKY (1965) (27), that the immune response of the host against tumor is probably altered from growth inhibitory effect to immunological enhancement. Just as a minute amount of serum antibody, though somewhat delayed in its appearance, intervenes the approach of sensitized lymphoid cells, such serum antibody would possibly make a coating on specific antigens on the surface of tumor cells.

However, as already mentioned in early part of this report with MC-induced sarcoma of early stage when no alteration would have occurred in cancer specific antigens, the inhibitory effect in vivo of the cancer-bearing host on the tumor growth was strong in the regional lymph-node cells obtained one to two weeks after tumor transplantation but no inhibitory
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In any event, it is noteworthy that even in the cancer-bearing animal destined to die of tumors, as a certain stage of cancer, it acts inhibitorily on the tumor growth through its lymphoid system. It seems that this antitumor activity is dependent upon such factors as the strength of new tumor antigens altered by cancerization, the potency and degree of antigenicity of the host, and the strength of surveillance mechanism of the host.

CONCLUSION

MC-induced sarcomas produced under the skin on the back between scapulas of C3H mice were transplanted successively to the mice of the same strain. Using the first and the second generation tumors, viable tumor cells were prepared and with these tumor cells C3H mice were inoculated. From these sensitized mice regional lymph nodes were taken out at certain intervals and lymph-node cells were prepared. These tumor cells were mixed with regional lymph-node cells in the ratio of 1:10, and the mixed cells were transplanted subcutaneously on the back of C3H mice, and the development and growth of tumors were observed at intervals. As a result it was found that the inhibitory effect of these regional lymph-node cells on the tumor growth was strong one to two weeks after the transplantation, but beyond 3, or 4 weeks no inhibition was observable.

In connection with the present in vivo experiments, some comments were made on the available literature, and it has been demonstrated that even in the cancer-bearing animal destined to die of tumors, at certain stage of cancer there is seen an inhibitory effect of the host on the tumor growth by way of the lymphoid system and that such a response of the host in vivo seems to be correlated well with in vitro reaction.

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