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In vitro studies on tumor-specific immunity by using C3H mammary cancer-A cells. I. Inhibitory effect of lymph-node cells from the tumor bearing isologous C3H mouse on the proliferation of the tumor cells*

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Abstract

As a link in the series of studies on tumor-specific immunity, in vitro inhibitory effect of sensitized isologous lymph-node cells on the proliferation of C3H mammary cancer was studied. For this purpose tissue culture was conducted with regional lymph-node cells obtained from truly isologous C3H mouse inoculated with A strain cells derived from C3H mouse mammary cancer along with A cells, and the following results were obtained. In the case of tissue culture with those lymph-node cells obtained from the groups of mice 10 days after the inoculation of 5×10^6 A cells, the inhibitory effect on the proliferation of A cells was most marked, followed by that of those taken on day 14, 7, and 5 decreasing in the order mentioned. In the case with those regional lymph-node cells obtained from mice which did not have recurrence of tumor 1 week after extirpation of 2-week old tumor, the inhibitory effect on proliferation of A cells was marked, with the regional lymph-node cells obtained two weeks after transplantation of 1×10^8 A cells there could be observed no inhibitory effect at all. This suggests that at a certain stage after implantation of such regional lymph- node cells there develops a specific anti-tumor activity in the host.

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**IN VITRO STUDIES ON TUMOR-SPECIFIC IMMUNITY
BY USING C3H MAMMARY CANCER-A CELLS**

**I. INHIBITORY EFFECT OF LYMPH-NODE CELLS
FROM THE TUMOR BEARING ISOLOGOUS C3H
MOUSE ON THE PROLIFERATION OF
THE TUMOR CELLS**

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KLEIN¹ established the concept of tumor specific immunity through his observation on the primary autochthonous host transplanted with methylcholanthrene-induced sarcoma. Likewise PREHN², by using dibenz-(a, h)-anthracene-induced tumor, and OLD³, using benzpyrene-induced tumor, demonstrated the existence of tumor-specific antigen. By using MH 134 cells TAKEDA⁴ reported the presence of anti-tumor activity of the lymphoid cells from the spleen of F₁ strain mice of C3H × dd sensitized with the tumor cells exposed to X-ray by which the tumor cells had lost the activity of cell division. These tumor-specific antigens *in vivo* have been demonstrated with isologous or autologous animals inoculated with viable tumor cells of the same strain after sensitization with previously treated tumor cells.

For *in vitro* determination of anti-tumor activity of sensitized lymphoid cells there are experiments by tissue culture method. HARA⁵, HANAOKA⁶ and ROSENAU^{7,8} observed a mutual cell damaging effect between lymphoid cells and target cells in such tissue culture, and they demonstrated a direct immune reaction between tumor cells and the lymphocytes of the host. However, as they all used homologous lymphocytes and antigenic cells, their experiments are in reality executed homotransplantation immunity *in vitro*.

In the present experiment regional lymph-node cells obtained from C3H mice sensitized by transplanting mammary cancer derived from the same strain mouse were made to act on target cells, the mammary cancer cells in tissue culture. As the result the existence of anti-tumor activity, the presence of tumor specific antigen, was demonstrated just as in homotransplantation immunity. Principal findings of the study are described in the following.

MATERIALS AND METHODS

Animals : C3H (H-2^k) female mice weighing about 20g whose genotype was clear were purchased from The Mouse Colony of Okayama University. They were fed on solid feed, MF of Oriental Yeast Company mixed with some fresh vegetables and grown to the age of 6~8 weeks old.

Sensitization of mice : These 63 animals were divided into 4 groups. To the first group (10 mice) 1×10^8 A strain cells and to the second group (10 mice) 5×10^6 cells were inoculated subcutaneously on the back between the scapula, and to the third group (15 mice) 5×10^6 of the same A cells were similarly inoculated. In the case of groups 1 and 2 regional lymph nodes of axilla were extirpated two weeks after the inoculation, and lymph nodes in group 3 were from 9 mice which did not have recurrence of tumor 1 week after extirpation of 2 week-old tumor. Group 4 was further subdivided into 4 subgroups of 7 animals each. To each of these subgroups 5×10^6 A strain cells were transplanted in the same manner as with the other groups, 14, 10, 7 and 5 days respectively before the extirpation of the axillary lymph nodes, and the lymph nodes were extirpated from all the subgroups simultaneously on the same day to be used for the experiment (Fig. 1).

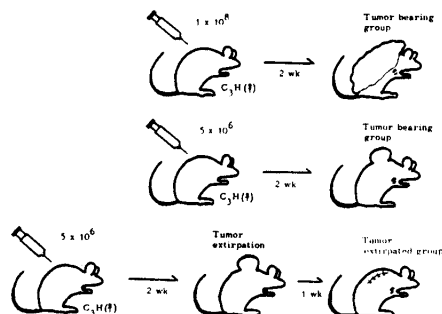


Fig. 1 Graphic Representation of Transplantation of C3H Mammary Cancer Cells

Tissue culture cells : A-strain cells are the culture cells originally derived from mammary cancer that grew spontaneously in C3H female mice and maintained in the Pathological Section of Okayama University Cancer Institute and these cells are first treated with 0.25% trypsin GKN solution and passed through the 150-mesh filter, and cultured in the medium of YLE (Earl's balanced salt solution containing 0.1% (w/v) yeast extract and 0.5% (w/v) lactalbumin hydrolysate) supplemented with 50% bovine serum.

Lymph-node cell suspension : From six cancer bearing animals each of the three groups mentioned above are selected at random and regional lymph

nodes are extirpated aseptically while under ether anesthesia. These serve as the materials for sensitized lymph-node cells of each group. From 15 untreated normal mice axillary lymph nodes are extirpated in a similar manner and these serve as the materials for control lymph-node cells. These extirpated lymph nodes are cut into small pieces with ophthalmic scissors and passed through 80-mesh filter. The filtrate is washed with cold Hank's solution by centrifugation at 2,000 rpm for 5 minutes and these washings are repeated three times. After removal of serum, the rest is suspended in 50% bovine serum plus YLE medium. Before making suspension in this instance, the countings of viable cells are taken by counting unstained cells by eosin Y staining⁹ and those showing over 80 per cent viable cells are used for the experiment.

Culture of lymph-node cells and isologous target cells: For each group, lymph-node cells, A strain cells, and penicillin are mixed in proportion of 16×10^6 /ml, 4×10^4 /ml, and 100 γ /ml, respectively to make the total volume 10 ml. One and half milliliters each of this mixture are pipetted into 6 short test tubes and the replicate cell culture is carried out at 37°C by the method of EVANS *et al.*¹⁰ At the intervals of 24 and 48 hours of incubation, 3 test tubes each are taken out and the medium is removed by gentle decantation. Then 1.5 ml of the crystal violet solution (containing 100 ml distilled water + 2.1 g citric acid + 50 mg crystal violet) are added to each of the three test tubes and the cells are incubated again at 37°C for 30 min. Next, the cells attached to the wall of test tube are detached gently by a rubber cleaner and by stirring gently a uniform cell suspension is made. A droplet of this suspension is placed on Bürker-Türk hemocytometer and the cell counts are taken more than six times for each test tube and the average of three test tubes is taken as the number of the increase in A strain cells. The distinction between lymph-node cells and A strain cells is easy because the former are hyperchromatic and have much smaller nucleus while the latter have larger nucleus.

RESULTS

Two weeks after the subcutaneous inoculation (on the back and in between the scapula) of A strain cells (derived from mammary cancer of C3H female mice) there developed tumors of $4.6 \times 3.5 \times 1.4$ cm in average size in the group 1 given 1×10^8 cells and $1.1 \times 0.9 \times 0.6$ cm in average in the group 2 inoculated with 5×10^6 cells. The regional lymph-node cells obtained from the group 1 did not show any inhibitory effect on A strain cells in culture (Fig. 2); the lymph-node cells from the group 2 did inhibit the proliferation of A cells; those lymph-node cells from group 3 obtained one week after the tumor extirpation, exhibited a marked inhibitory effect (Fig. 3).

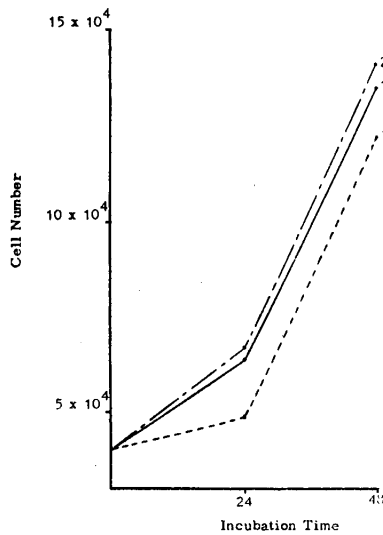


Fig. 2 Curves showing the proliferation of A strain cells in the presence of regional lymph-node cells obtained 2 weeks after inoculation of 1×10^8 A cells
 Note: 1 denotes control lymph-node cells+A cells, 2 denotes sensitized lymph-node cells+A cells, and 3 denotes A cells alone.
 For method, refer to the text.

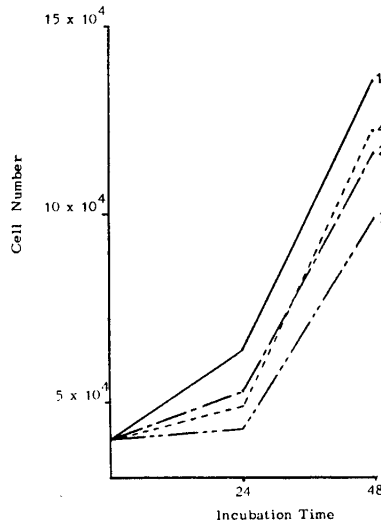


Fig. 3 Curves showing inhibitory effect of the regional lymph-node cells obtained 2 weeks after transplantation of 5×10^6 A cells and the regional lymph-node cells obtained one week after extirpation of 2-week old tumor, on A strain cells
 Note: 1 denotes control lymph-node cells+A strain cells, 2 (5×10^6) sensitized lymph-node cells+A strain cells, 3 (tumor extirpated) sensitized lymph-node cells+A strain cells, and 4 A strain cells alone.
 For method, refer to the text.

As for group 4 of the regional lymph-node cells extirpated at various intervals after the inoculation of 5×10^6 cells, those obtained on the tenth day showed the most marked inhibitory effect on the growth of A cells in culture, and such inhibitory effect grew weaker in the regional lymph-node cells in the order of day 14, 7, and 5 after the inoculation (Fig. 4). Looking at the data in this

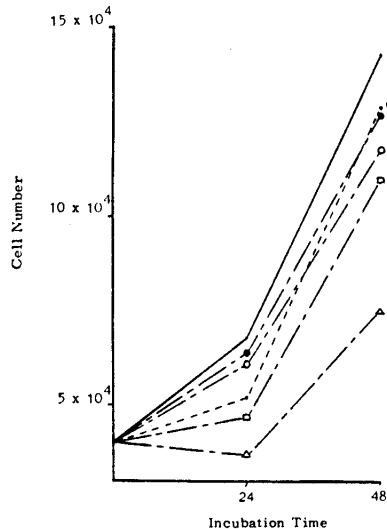


Fig. 4 Curves showing growth-inhibition effect of regional lymph-node cells from the animal inoculated with A strain cells on the A strain cells in tissue culture

Note: 1 denotes control lymph-node cells+A strain cells, 2 denotes (14 days later) sensitized lymph-node cells+A cells, 3 denotes (10 days later) sensitized lymph-node cells+A cells, 4 denotes (7 days later) sensitized lymph-node cells+A cells, 5 denotes (5 days later) sensitized lymph-node cells+A cells, and 6 denotes A strain cells alone.

For the method, refer to the text.

series of experiments, it is obvious that, compared with the tissue culture of A strain cells alone, those A strain cells cultured with untreated normal lymph-node cells invariably tend to accelerate the cell proliferation.

DISCUSSION

HIRSCH¹¹ demonstrated that the survival time of the inbred mice sensitized with spontaneous mammary cancer is prolonged but the animals die of tumor later. In his experiment with methylcholanthrene-induced sarcoma, KLEIN¹ challenged the autochthonous mice, together with groups of isologous animals, pre-treated with irradiated sarcoma cells, increasing doses of viable cells from the original MC-induced sarcoma and found the resistance against methylcholanthrene-

induced sarcoma not only in the primary autochthonous host but also in the isologous mouse. RIGGINS *et al.*¹² confirmed the immune reaction against spontaneous mammary cancer of C3H mice, and demonstrated that this reaction is weaker than that of methylcholanthrene-induced sarcoma, and the immune reaction has specificity to immunizing tumor, the extent of which is related to the inoculating period of tumor cells. In such a way the existence of tumor specific antigen *in vivo* has been made clear. In addition, KLEIN¹³ demonstrated that the lymph-node cells of isologous preimmunized mice inhibit the growth of methylcholanthrene-induced sarcoma in C57BL strain mice.

On the basis of these findings in the present experiments observations of the mutual reactions between target cells and sensitized lymph-node cells were carried out *in vitro*. As the result it has been clarified that in the regional lymph-node cells obtained from the truly isologous mice to which transplantation of A strain cells derived from C3H mouse was successful there appears anti-tumor activity at least in a certain stage after the inoculation.

While there seems to be no report like the present experiment where the regional lymph-node cells of the host in the case of isograft transplantation show anti-tumor activity to antigenic tumor cells, ROSENAU^{7,8} reported that lymph-node cells obtained from BALB/c strain mouse sensitized with L cells derived from C3H mouse in the absence of complement adhered specifically to L-cells and these L-cells were gradually damaged. HARA⁵, in his mixed cell tissue culture of JTC-11 strain cells (derived from Ehrlich tumor) and the corresponding sensitized lymph-node cells, found that the growth of JTC-11 cells was inhibited. BRONDZ¹⁴ showed that immune mouse lymphocytes exhibit cytotoxic effect on SaI cells. Everyone of these shows the action behaviors of antigenic cells at homotransplantation, and the action behaviors of sensitized lymphoid cells *in vitro* seem to represent the action of the host lymph-node cells against excess histocompatibility antigen of homologous transplant *in vivo*.

From the fact that the skin graft transplantation between C3H mouse (used in this experiment) survived permanently in place and there could be recongnized no immunological change at all¹⁵, it is assumed that C3H mouse is isologous as far as histocompatibility gene is concerned. Consequently, it might be fairly reasonable to assume that the anti-tumor activity by the regional lymph node to mammary cancer derived from isologous mouse is a reflection of immunological reaction *in vitro* of lymph-node cells to mammary cancer cell specific antigen other than histocompatibility gene itself.

Those regional lymph-node cells from the group extirpated of tumor show much stronger inhibitory action than the tumor bearing group, and those lymph-node cells from the group of mice on the threshold of death from tumors do not show any inhibitory action.

This fact seems to indicate that the lymph-node cells in the terminal stage of cancer become paralytic because of an enormous amount of antigens from tumor cells and react as a sort of enhancement factor to tumor. In either case, these two factors seem to be involved in the unlimited growth of cancer.

SUMMARY

As a link in the series of studies on tumor-specific immunity, *in vitro* inhibitory effect of sensitized isologous lymph-node cells on the proliferation of C3H mammary cancer was studied. For this purpose tissue culture was conducted with regional lymph-node cells obtained from truly isologous C3H mouse inoculated with A strain cells derived from C3H mouse mammary cancer along with A cells, and the following results were obtained.

In the case of tissue culture with those lymph-node cells obtained from the groups of mice 10 days after the inoculation of 5×10^8 A cells, the inhibitory effect on the proliferation of A cells was most marked, followed by that of those taken on day 14, 7, and 5 decreasing in the order mentioned.

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