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In vitro studies on the inhibitory effect of lymphoid cells. II. Antitumor activity of lymphoid cells from spontaneous mammary tumor-bearing mice on the autochthonous primary culture tumor cells*

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Abstract

As a step in the elucidation of human cancer immunity we studied antitumor activity of lymphoid cells by conducting a series of cultures using the primary culture of cells from spontaneous mammary cancers from C3H and RIll mice mixed with autochthonous lymphoid cells, and obtained the following results. 1) With 24 mammary tumors obtained from 24 mammary cancer. bearing mice, we prepared 22 suspensions containing sufficient numbers of free tumor cells, and attempted primary culture with them. As a result we were able to attain satisfactory primary culture cells in 18 trials. 2) With each group of the 18 primary culture tumor cells we conducted mixed cultures with autochthonous lymphoid cells (mainly spleen cells) in proportion of 1 : 40, for 48 hours, and counted viable tumor cells after the culture. As a result it was found that in 11 trials the lymphoid cells showed antitumor activity. In the remaining 7 groups of lymphoid cells there could be observed no antitumor activity, but some of them showed tendency to slightly accelerate the growth of tumor cells. 3) On looking at the correlation between the antitumor activity of lymphoid cells and the ratio of tumor weight/body weight, it was revealed that the antitumor activity is greatest when the tumor is around 10% the body weight, and as the tumor grows larger, such antitumor activity disappears. From these results, it may be concluded that even in spontaneous mammary cancer of mouse, autochthonous lymphoid cells exhibit anti. tumor activity on indigenous tumor, and this seems to indicate that cell. mediated immunity has been established.

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IN VITRO STUDIES ON THE INHIBITORY EFFECT OF LYMPHOID CELLS.
II. ANTITUMOR ACTIVITY OF LYMPHOID CELLS FROM SPONTANEOUS MAMMARY TUMOR-BEARING MICE ON THE AUTOCHTHONOUS PRIMARY CULTURE TUMOR CELLS

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As reported in a previous paper (1), we have demonstrated that in our time-lapse observations on the antitumor activity of regional lymph nodes *in vitro* after isotransplantation of methylcholanthrene-induced sarcoma to Zb mice (C3H mice without mammary tumor virus), these host regional lymph nodes exhibit antitumor activity just as in the case of homotransplantation of tumor (2), and such an antitumor activity decreases when the tumor grows beyond a certain size and finally disappears. It has been already clarified by several reports that methylcholanthrene-induced sarcoma possesses tumor specific transplantation antigen (TSTA) (3, 4), and *in vitro* the antitumor activity of host mouse lymphocytes seems to be directed to this TSTA (1, 5).

Concerning spontaneous tumors there are only a few reports that have identified tumor specific antigen, and even with spontaneous mammary cancers, aside from the report by ATTIA *et al.* (6) there are only a few reports on the demonstration of mammary cancer specific antigen (7, 8). Even with spontaneous tumors most of the available reports are concerned with tumor transplantation experiments to identify TSTA *in vivo* (9), hence such a method is not applicable to the demonstration of human tumor antigens.

Here we present the results of our mixed cultures of the primary culture cells obtained from spontaneous mouse mammary cancer added with lymphoid cells of host tumor-bearing mouse to detect antitumor activity of these lymphoid cells, in which we have been able to demonstrate the establishment of cell-mediated immunity.

MATERIALS AND METHODS

Experimental animals: The animals used were 17 C3H mice, 7 RIII mice, bearing spontaneous mammary cancer, 20 normal C3H mice, and 10 RIII mice, all weighing 25-36 g and over the age of 6 months old, purchased from the Mouse Colony of Okayama University. They were fed on solid feed, Oriental Yeast Company, mixed with some vegetables for at least 1 week before experimental use, and their body weight was measured just before the experiment.

Primary tumor cell culture: Mammary tumor grown to the size of thumb, is excised, weighed and free tumor cell suspensions are prepared by MADDEN-BURK method (10). Namely, the tumor taken out asceptically is sliced into thin sections, and to 1 g tumor section is added 8 ml cold Hank's solution+10 ml 0.20% trypsin+6-7 drops of 0.04% DNase, and is left standing for 2 hours while stirring occasionally. The tumor cell suspension so prepared is then filtered through 80-mesh filter, and over these cells attaching to the mesh GKN and DNase solutions are poured, filtered for another one hour, while stirring, and the filtrate thus obtained is used as culture cells. The filtrate is then cultured in the medium containing 20% calf serum plus YLE solution.

At first the number of viable tumor cells is determined by the eosin dye exclusion test, then the number of viable tumor cells is adjusted to $5\text{-}30\times 10^4$/ml, 1.5 ml each of the cell suspension is put separately into each test tube, and the replicate culture is conducted at 37°C for 3 days. Those cells having large nucleus and distinct nucleoles with thin chromatin structure are taken as tumor cells, and their number is counted.

Lymphoid cell suspension: The spleen or axillary lymph nodes are taken out asceptically from the mice bearing mammary cancer and from normal mice of the same strain. The spleens and lymph nodes so taken out are cut into small slices with ophthalmic scissors in cold Hank's solution, and filtered through 80-mesh filter. Each of these filtrates is washed, centrifuged, suspended in the YLE medium with calf serum.

Addition of lymphoid cells to primary tumor cell culture: After 3-day culture of tumor cells in short test tubes, several test tubes are taken out at random, its supernatant is discarded, 1.5 ml of crystal violet solution is then added to each tube, and again incubated at 37°C for 30 minutes. Next those cells attached on the vessel wall are scraped off gently with a rubber cleaner, and they are stirred well to make a uniform cell suspension. Taking a drop of this cell suspension into Bürker-Türk hemocytometer the number of viable tumor cells is determined by the number of cell nuclei.

At the same time the number of viable tumor cells after 3-day culture is confirmedto be uniform in the cell suspension. Then the remaining short test tubes are divided into several groups of 3-6 tubes each, after decanting supernatant to each respective group of test tubes is added 1.5 ml of lymphoid cell suspension of the mouse bearing tumor or 1.5 ml of lymphoid cell suspension of normal mouse of the same strain (tumr cell number : lymphoid cell number = 1 : 40), and further stationary culture is carried out at 37°C for 48 hours. At the end of culture time

crystal violet solution is put into the test tubes to determine the number of viable tumor cells in each test tube. In this instance, the average number of 3-6 test tubes of each group is taken as the actual viable tumor cells of respective group.

RESULTS

With free cells prepared from 24 different mammary cancers from 24 cancer-bearing mice the successful cases of primary culture proved to be 18, of which 13 cases were the mammary cancers from C3H mice and another from RIII mouse. Six of them resulted in failure, of which 2 cases failed because of insufficient number of tumor cells, and the other 3 cases because of too small number of the cells attaching to the culture vessel wall, and one because of infection. The antitumor activity of cancer-bearing C3H mouse lymphoid cells against autochthonous tumor cells is as shown in Table 1. As can be seen in this table, among 13 trials, those host lymphoid cells showing antitumor activity prove to be 7 cases, and 6 cases do not show any antitumor activity. The antitumor activity of cancer-bearing RIII mouse lymphoid cells is shown in Table 2. Host lymphoid cells with antitumor activity are 4 cases out of 5. Even so, there can be observed tendency to slightly accelerate proliferation of tumor cells in cases of addition of autochthonous lymphoid cells without antitumor activity. By conducting T-test of the mixed culture supplemented with lymphoid cells from normal mouse of the same strain in which the num-

TABLE 1 EFFECTS OF LYMPHOID CELLS ON PRIMARY CULTURE TUMOR CELL FROM C3H MICE

No.	body weight (g)	tumor weight (g)	tumor weight / body weight	Cancer cell number after 48-hr culture (1) Tumor alone	(2) Tumor + autochthonous lymphoid cells	(3) Tumor + isogeneic lymphoid cells	Inhibition (%) (2)/(3)	P.
1	30.0	3.2	0.106	11.2×10^4	8.6×10^4	10.5×10^4	81.4%	<0.05
2	32.4	3.1	0.095	16.5	14.4	17.3	83.2	<0.05
3	29.2	2.7	0.092	6.4	5.4	6.7	80.6	<0.05
4	31.5	3.0	0.095	4.2	3.4	4.4	77.3	<0.01
5	32.0	3.3	0.103	4.8	3.4	4.7	72.4	<0.02
6	30.2	3.9	0.129	7.2	5.5	6.8	80.9	<0.05
7	29.8	3.2	0.107	10.3	8.3	10.1	82.1	<0.05
8	25.2	3.5	0.134	4.6	4.3	4.0	107.5	>0.1
9	28.8	4.1	0.142	6.2	5.9	5.6	105.4	>0.1
10	28.5	2.0	0.070	4.7	4.8	4.2	114.3	<0.05
11	30.4	4.5	0.149	8.3	8.4	8.0	105.0	>0.1
12	31.7	6.7	0.212	4.4	4.0	4.5	88.8	>0.1
13	30.4	3.8	0.125	6.6	6.0	6.4	93.7	>0.1

TABLE 2 EFFECTS OF LYMPHOID CELLS ON PRIMARY CULTURE TUMOR CELLS FROM RIII MICE

No.	body weight (g)	tumor weight (g)	tumor weight / body weight	cancer cell number after 48-hr culture (1) Tumor alone	(2) Tumor + autochthonous lymphoid cells	(3) Tumor + isogeneic lymphoid cells	Inhibition (%) (2)/(3)	P.
1	26.2	3.1	0.114	9.8×10^4	8.0×10^4	10.4×10^4	76.9%	<0.05
2	28.0	3.5	0.125	10.9	7.6	10.2	74.5	<0.05
3	36.5	3.6	0.093	4.4	3.0	4.8	62.5	<0.05
4	26.0	2.8	0.108	6.5	4.8	7.0	68.6	<0.01
5	27.8	3.5	0.126	8.4	8.0	7.8	102.5	>0.1

ber of viable tumor cells is taken as the standard, the probability (P) of experitmental error is calculated. In correlation between the ratio of weight of the excised tumor to the body weight and the antitumor activity of lymphoid cells, we find that when the tumor weighs more or less 10% of the body weight, there can be observed antitumor activity in 9 cases out of 11, but when the tumor has grown over 12% the body weight, the antitumor activity weakens in 6 cases out of 7 (Tables 1, 2).

DISCUSSION

In the past ten years tumor specific immunity has been demonstrated in various animal cancers, especially in chemical or virus-induced tumor, but the identification of spontaneous tumor specific transplantation antigen has resulted in failure and it has been thought for a long time that spontaneous tumors including human cancers lack tumor specific transplantation antigen. In the spontaneous mammary cancer of C3H mice likewise it has been demonstrated that in the mice naturally carrying mammary tumor virus no immune response to tumor specific transplantation antigen is elicited and such mice do not acquire antitumor activity against spontaneous mammary cancer (11, 12), but it has been clarified that the mice without mouse tumor virus are immunized against this tumor specific transplantation antigen (13). In contrast, in the present experiments we have been able to demonstrate that spontaneous mammary cancer cells prepared from the mice thought to possess natural mouse tumor virus do at least reveal antitumor activity, when such tumor cells are previously subjected to primary culture and then cultivated with autochthonous host lymphoid cells. Recently, SATOH (5) of our laboratory has reported that when A-cells, an established cell line derived from spontaneous mammary cancer of C3H mice, are isotransplanted to C3H mice

having mouse tumor virus, regional lymph node cells of the host act specifically to inhibit the proliferation of A-cells *in vitro*. HEPPER and PIERCE (13) have also demonstrated that the colony formation of mouse mammary cancer cells *in vitro* is inhibited on the addition of autochthonous lymph node cells of the cancer-bearing host. In the present study we have measured the proliferation of tumor cells by their nuclear counts and have then represented quantitatively the antitumor activity of autochthonous lymphoid cells. In the transplantation *in vivo* it is difficult to detect tumor specific transplantation antigen, but *in vitro* it can be demonstrated in some instances.

Successful instances of primary culture with excised mammary cancers proved to be 18 out of 24 trials, of them only in one trial regional lymph node cells were used as lymphoid cells. Since it is very difficult to obtain sufficient number of regional lymph node cells from one mouse, we used spleen as the source of lymphoid cells in the rest of experiments. It is quite simple to differentiate lymphocytes from tumor cells by the cell size, the shape of nucleus and the color tone.

When the tumor grows to a certain size after spontaneous development or after transplantation, regional lymph node cells proximal to the tumor acquires antitumor activity, and this activity gradually becomes stronger with lapse of time. However, when the tumor grows beyond a certain size, the antitumor activity of regional lymph node cells decreases, while there develops antitumor activity in more distant lymph nodes and lymphatic tissues. Such a tendency has been demonstrated not only in isotransplantation of methylcholanthrene induced tumor (5), but also in homotranrplantation of Ehrlich ascites tumor (2), in the isotransplantation of A-cells (a cell line derived from C3H mouse mammary cancer cells) as well as in human cancer. In the present experiment as we did not know clearly the onset time of the spontaneous mammary cancer in mice that we used, we determined the presence or the absence of antitumor activity by the ratio of the tumor weight to the body weight of animal. It has been found that the antitumor activity seems to decrease when the tumor grows over 12% the body weight. In addition, after the onset of tumor only when the tumor reaches a certain size it is thought that there appears antitumor activity against tumor specific transplantation antigen, but one out of the 7 cases that did not show antitumor activity revealed a relatively small ratio (tumor weight/body weight) as 0.07.

As it has been generally considered strongly that cancer immune reaction cannot occur in the autochthonous mammary cancer host because the cancer-bearing host, having mouse tumor virus from its birth,

is in a state of a immunological tolerance, it seems to be an important finding that cell-mediated immunity has been extablished in the present experiment, and this finding points strongly to a possibility of establishing immunity in spontaneous cancers including human cancers.

SUMMARY

As a step in the elucidation of human cancer immunity we studied antitumor activity of lymphoid cells by conducting a series of cultures using the primary culture of cells from spontaneous mammary cancers from C3H and RIII mice mixed with autochthonous lymphoid cells, and obtained the following results.

1) With 24 mammary tumors obtained from 24 mammary cancer-bearing mice, we prepared 22 suspensions containing sufficient numbers of free tumor cells, and attempted primary culture with them. As a result we were able to attain satisfactory primary culture cells in 18 trials.

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From these results, it may be concluded that even in spontaneous mammary cancer of mouse, autochthonous lymphoid cells exhibit anti-tumor activity on indigenous tumor, and this seems to indicate that cell-mediated immunity has been established.

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