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Time-lapse changes in the concomitant immunity of lymphocytes from different sites of mice isografted with methylcholanthrene-induced tumor.

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Abstract

As a step in the elucidation of the mutual relationship between the degree of cancer progress and the antitumor activity of lymphocytes from different sites in cancer-bearing body, we isografted methylcholanthrene-induced tumor (MC-tumor) subcutaneously on the back of mice. The regional axillary lymph nodes, spleen and distant mesenteric lymph nodes were removed from these animals one, two, three, and four weeks later. We mixed lymphocytes prepared from these lymphatic tissues with primary MC-tumor culture cells and cultured together to estimate antitumor activity of lymphocytes from different sites. It has been found that a strong antitumor activity can be seen only in those regional axillary lymph node cells taken out one or two weeks after tumor transplantation and such an activity is weakened by three or four weeks. On the other hand, distant mesenteric lymph node cells one or two weeks after the transplantation have no antitumor activity as yet, while at the terminal cancer stage of four weeks there appears a stronger antitumor activity than that of regional lymph nodes. In the spleen, a strong antitumor activity can be observed in the third week after tumor transplantation, but the activity disappears by the fourth week. These findings support our previous findings in that for the tumor onset after the transplantation the antitumor activity seems to appear first in the regional lymph nodes, and when the tumor grows beyond a certain size, such an activity diminishes while it appears in further distant lymphatic tissues.

KEYWORDS: Time-lapse changes, different sites

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**TIME-LAPSE CHANGES IN THE CONCOMITANT
IMMUNITY OF LYMPHOCYTES FROM DIFFERENT
SITES OF MICE ISOGRAFTED WITH METHYL-
CHOLANTHRENE-INDUCED TUMOR**

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Abstract: As a step in the elucidation of the mutual relationship between the degree of cancer progress and the antitumor activity of lymphocytes from different sites in cancer-bearing body, we iso-grafted methylcholanthrene-induced tumor (MC-tumor) subcutaneously on the back of mice. The regional axillary lymph nodes, spleen and distant mesenteric lymph nodes were removed from these animals one, two, three, and four weeks later. We mixed lymphocytes prepared from these lymphatic tissues with primary MC-tumor culture cells and cultured together to estimate antitumor activity of lymphocytes from different sites. It has been found that a strong antitumor activity can be seen only in those regional axillary lymph node cells taken out one or two weeks after tumor transplantation and such an activity is weakened by three or four weeks. On the other hand, distant mesenteric lymph node cells one or two weeks after the transplantation have no antitumor activity as yet, while at the terminal cancer stage of four weeks there appears a stronger antitumor activity than that of regional lymph nodes. In the spleen, a strong antitumor activity can be observed in the third week after tumor transplantation, but the activity disappears by the fourth week. These findings support our previous findings in that for the tumor onset after the transplantation the antitumor activity seems to appear first in the regional lymph nodes, and when the tumor grows beyond a certain size, such an activity diminishes while it appears in further distant lymphatic tissues.

Even in the tumor-bearing body destined to die of tumor, if left untreated, we have demonstrated the presence of cell-mediated concomitant immunity which acts inhibitorily on its own tumor, by the specific antitumor activity *in vitro* as the parameter. However, in the case of a progressive cancer, be it transplanted cancer, spontaneous mammary cancer in mouse or human cancer, the antitumor activity of lymphocytes of the tumor-bearing body is diminished (1-6). In our previous study on the antitumor activities of the regional axillary lymph nodes, spleen and distant mesenteric lymph

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nodes of the mice conducted along with the lapse of time after homografting Ehrlich cancer cells subcutaneously on the back, we clarified that there is some difference in the antitumor activity of lymph node cells of various sites due to the degree of cancer advance, and that the antitumor activity of the regional lymph nodes appears as the tumor proliferates, but in the advanced stage of cancer the antitumor activity of regional lymph nodes diminishes, while the antitumor activity appears in the distant mesenteric lymph nodes (7). In the present report we describe the results of isografting methylcholanthrene-induced tumor (MC-tumor) that have confirmed our previous findings obtained after homografting of Ehrlich ascites tumor.

MATERIALS AND METHODS

Animals: The animals used were C3H mice of 6-10 weeks old, weighing about 22 g. They were fed on solid food, MF of Oriental Yeast Company (Japan).

Tumor cells: One mg of 20-methylcholanthrene suspended in arabian gum was injected to C3H mice subcutaneously on the back. Tumors grown to 1-2 cm in size at the injection site are excised aseptically about three months later, and 1 mm³-piece of such a tumor is isografted subcutaneously on the back of C3H mice. Such a transplantation is successively carried out at intervals of three weeks to maintain the tumor in animals. For the experiment, tumors obtained from the mice of the sixth to eighth generations were used.

Primary culture of tumor cells: The tumor grown to 1-2 cm in diameter is removed aseptically, and tumor cell suspension is prepared by almost the same method as that of MADDEN-BURK (8). To each one gram of finely sliced tumor section 8 ml of Hanks' solution, 10 ml of 0.2% trypsin, 0.3 ml of 0.04% DNase are added, and left standing for 90 min. Then discarding the supernatant the same amounts of Hanks' solution, trypsin and DNase are added, and the mixture is stirred with a magnetic stirrer for 60 min. Next the mixture is filtered through a 150-mesh filter, and the filtrate is centrifuged at 1,000 rpm for 10 min. To the residue is added 30 ml TC-199, and after stirring well it is centrifuged at 1,000 rpm for 10 min, by discarding the supernatant 20% calf serum and TC-199 (containing 30 μ /ml of cephalothin) are added to the residue to prepare tumor cell suspension as to contain 30×10^4 cells per one ml. Those cells with a large nucleus, bright nucleoli and a light nuclear architecture are taken as tumor cells. By placing 2 ml of such tumor cell suspension each into 50 separate short test-tubes the stationary replicate culture is carried out at 37°C for three days.

Tumor transplantation: To 40 mice MC-tumor piece 1 mm³ in size is transplanted subcutaneously on the back between scapulae.

Lymphocytes suspension: At one, two, three and four weeks after tumor transplantation, 5-6 mice each, having tumors of the same size, are selected at random, and under ether anesthesia regional axillary lymph nodes, spleen

and distant mesenteric lymph nodes are taken out from these mice. As the control, axillary lymph nodes are collected from normal mice. Excised lymph nodes and spleen are cut into small fragments and passed through an 80-mesh filter. To the filtrate so obtained cold Hanks' solution is added, and it is washed by centrifuging at 1,500 rpm for 5 minutes. Such a washing is repeated thrice, and the cells are suspended in the culture medium identical with the medium used for tumor cell suspension. The cell suspensions are prepared so as to contain over 95% of viable small lymphocytes.

Mixed culture of primary MC-tumor culture cells with lymphocytes: After 3-day culture of tumor cells several short test tubes are selected at random, discarding supernatant 2.0 ml of crystal violet solution (100 ml distilled water plus 2.1 g citric acid plus 50 mg crystal violet) is added to each tube, and warmed to 37°C for 30 min. Then the cells adhering to the vessel wall are gently scraped off, shaken well to make a uniform cell suspension, and the nucleus counts are taken with a hemocytometer to calculate the average number of tumor cells as well as to confirm that tumor cells are proliferating equally well in each short test tube. The remaining short test tubes are then divided into five groups; one culture group of tumor cells alone without lymphocyte addition, one control group added with normal mouse lymphocytes, one culture group added with regional axillary lymph node cells, one culture group added with spleen cells, and the last culture group added with distant mesenteric lymph node cells. Supernatant is discarded from every group, to the culture group of tumor cells alone 2.0 ml of culture medium is added, and to the control groups as well as to the test groups also 2.0 ml each of lymphocyte solution containing 20-fold the number of tumor cells adjusted on the experimental day is added, and cultured further for 48 or 72 hours at 37°C. At the termination of 48-hour culture or 72-hour culture, 4-5 short test tubes are picked up from each group, and the crystal violet solution is added to each test tube, then the number of tumor cells is counted as already described, and the average numbers counted here are taken as the tumor-proliferation number in each group at this instant.

RESULTS

1. *Antitumor activity in the first week after tumor transplantation:* A significantly strong antitumor activity can be observed in the regional axillary lymph node cells but no antitumor activity can at all be recognized in both the spleen and the distant mesenteric lymph nodes (Table 1).

2. *Antitumor activity in the second week after tumor transplantation:* Regional axillary lymph nodes show an antitumor activity in the second week after tumor transplantation, but by 72-hour culture the distant mesenteric lymph nodes reveal only a slight antitumor activity. In spleen, no activity appears (Table 2).

3. *Antitumor activity in the third week after tumor transplantation:* By the third week after tumor transplantation no significant antitumor activity

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TABLE 1 ANTITUMOR ACTIVITY IN THE FIRST WEEK AFTER TUMOR TRANSPLANTATION

Lymphocytes	No. of tumor cells after culture (Mean $\pm$ S.E. *)		t-test
	48 hr	72 hr	
Regional axillary lymph node	14.4 $\pm$ 1.44	12.2 $\pm$ 1.25	p<0.001
Distant mesenteric lymph node	17.8 $\pm$ 2.40	20.9 $\pm$ 1.77	p>0.9
Spleen	15.0 $\pm$ 1.47	18.3 $\pm$ 1.62	0.4>p>0.3
Normal lymph node (control)	16.2 $\pm$ 1.96	21.1 $\pm$ 1.66	
Tumor cell alone	20.4 $\pm$ 1.62	22.8 $\pm$ 1.77	

* $\times 10^4$

TABLE 2 ANTITUMOR ACTIVITY IN THE SECOND WEEK AFTER TUMOR TRANSPLANTATION

Lymphocytes	No. of tumor cells after culture (Mean $\pm$ S.E. *)		t-test
	48 hr	72 hr	
Regional axillary lymph node	17.2 $\pm$ 2.38	19.0 $\pm$ 2.13	0.5>p>0.4
Distant mesenteric lymph node	20.9 $\pm$ 1.47	21.7 $\pm$ 1.72	0.9>p>0.8
Spleen	23.5 $\pm$ 1.51	24.4 $\pm$ 1.47	p>0.9
Normal lymph node (control)	18.7 $\pm$ 2.10	24.8 $\pm$ 2.18	
Tumor cell alone	21.6 $\pm$ 1.48	26.2 $\pm$ 4.78	

* $\times 10^4$

TABLE 3 ANTITUMOR ACTIVITY IN THE THIRD WEEK AFTER TUMOR TRANSPLANTATION

Lymphocytes	No. of tumor cells after culture (Mean $\pm$ S.E. *)		t-test
	48 hr	72 hr	
Regional axillary lymph node	16.9 $\pm$ 1.56	21.4 $\pm$ 1.88	p>0.9
Distant mesenteric lymph node	17.5 $\pm$ 2.50	18.8 $\pm$ 1.47	0.6>p>0.5
Spleen	14.1 $\pm$ 1.32	13.0 $\pm$ 1.39	p<0.001
Normal lymph node (control)	17.1 $\pm$ 1.45	20.9 $\pm$ 1.70	
Tumor cell alone	18.1 $\pm$ 1.28	23.0 $\pm$ 2.61	

* $\times 10^4$

can be observed in either regional or distant lymph nodes, but a strong activity can be seen in spleen (Table 3).

4. *Antitumor activity in the fourth week after tumor transplantation:* A slight antitumor activity can be observed in the regional lymph nodes and spleen but the strongest antitumor activity appears in the distant mesenteric lymph nodes (Table 4).

TABLE 4 ANTITUMOR ACTIVITY IN THE FOURTH WEEK AFTER TUMOR TRANSPLANTATION

Lymphocytes	No. of tumor cells after culture (Mean $\pm$ S.E. *)		t-test
	48 hr	72 hr	
Regional axillary lymph node	11.5 $\pm$ 1.55	12.3 $\pm$ 1.75	0.3 > p > 0.2
Distant mesenteric lymph node	11.0 $\pm$ 1.61	9.1 $\pm$ 1.56	0.01 > p > 0.001
Spleen	6.2 $\pm$ 1.29	13.1 $\pm$ 1.37	0.4 > p > 0.3
Normal lymph node (control)	12.8 $\pm$ 1.34	15.4 $\pm$ 1.40	
Tumor cell alone	13.5 $\pm$ 1.69	17.6 $\pm$ 1.69	

* $\times 10^4$

DISCUSSION

Most of the studies (9, 10) on the cellular concomitant immunity in the cancer-bearing body in the past have dealt with the antitumor activity of peripheral blood lymphocytes to primary cultured autochthonous tumor cells or to established cells, and there are hardly any investigations on the antitumor activity of lymph node cells, especially on the antitumor activity of lymphatic tissues according to different sites of the body. For the past ten years we have been studying the correlation between changes in antitumor activity of lymph node cells according to sites in the cancer-bearing body and the advance of cancer, using the antitumor activity *in vitro* of lymphocytes to corresponding tumor cells as parameter. HARA (1) in his homografting experiments of Ehrlich cancer cells, and SATOH (2) in isografting of a cell strain derived from C3H mammary cancer cells, have observed that by 10–14 days after the transplantation there appears a very strong antitumor activity in regional lymph nodes, but after lapse of 14 days the antitumor activity of lymph nodes diminishes along with growth of tumor in size. OHSUGI (5) has observed that using mice bearing spontaneous mammary cancer the antitumor activity of spleen cells clearly diminishes when the mammary cancer grows 10% over the body weight, and ORITA *et al.* (6) have conducted a series of tissue cultures of the primary culture cells derived from surgically extirpated human cancers such as gastric cancer and mammary cancer mixed with autochthonous lymph node cells extirpated in operation. In the 30 series of their experiments they state that 21 cases out of 30 (70%) lymph

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node cells showed an antitumor activity on the primary culture cells, but in comparing the antitumor activity of lymph nodes according to sites such as regional lymph node and distant mesenteric lymph node they state that the regional lymph nodes showed antitumor activity in 7 out of 13 cases (54%) and distant lymph nodes in 14 out of 17 (82.4%), indicating that there is a clear-cut difference in the antitumor activity of lymph nodes according to sites even in man.

Such a time-lapse change in the antitumor activity of regional lymph nodes is clearly seen even in the transplantation neutralization tests *in vivo*. For an instance, KASHIHARA (11) isografted MC-induced tumor subcutaneously on the back of mice and collected regional axillary lymph nodes from these animals with lapse of days after the isografting, and conducted the neutralization experiments by transplanting MC-induced tumor cells mixed with 10-fold number of regional axillary lymph node cells subcutaneously on the back of untreated normal mice and found that regional lymph nodes one to two weeks after such a transplantation clearly showed antitumor activity, but the lymph nodes collected three, four weeks later; namely, in the middle and terminal stage, had no longer any antitumor activity. Changes in the antitumor activity of lymphatic tissues at various sites of the body were previously described by homografting experiments with Ehrlich cancer cells, and in the present report the results of isografting of MC-induced tumor are stated. Considering the report of FISHER and FISHER (12) in which they state, that in the case where tumor is transplanted to a mouse from which regional lymph nodes were previously removed, the onset of antitumor activity is delayed and the intensity of antitumor activity is less, it may be said that the onset of tumor and antitumor activity would appear in the regional lymph nodes after tumor transplantation, and the antitumor activity diminishes as tumor grows larger and such an activity appears in more distant lymphatic tissues instead. It seems that the fall in antitumor activity of regional lymph nodes in progressive cancer is due mainly to an inflow of excess tumor-associated antigens from tumor to regional lymph nodes; namely, mainly centering around the mechanisms of so-called high zone paralysis.

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