

Acta Medica Okayama

Volume 24, Issue 4

1970

Article 6

AUGUST 1970

In vitro studies on the inhibitory effect of
lymph-node cells. I. Antitumor activity of
regional lymph-node cells from
methylcholanthrene-induced sarcoma bearing
mice on the same primary culture sarcoma
cells

Masaki Ohsugi*

*Okayama University,

Copyright ©1999 OKAYAMA UNIVERSITY MEDICAL SCHOOL. All rights reserved.

In vitro studies on the inhibitory effect of lymph-node cells. I. Antitumor activity of regional lymph-node cells from methylcholanthrene-induced sarcoma bearing mice on the same primary culture sarcoma cells*

Masaki Ohsugi

Abstract

As a step in the elucidation of immunity of human cancer from the standpoint of homotransplantation immunity, we conducted mixed cultures of regional lymph node cells from C3H mouse isografted with methylcholanthrene-induced sarcoma (MC-tumor) together with the primary culture MC-tumor cells, and observed the behaviors of these lymph node cells to the MC-tumor cells and compared the effects of these lymph node cells with those of normal mouse lymph node cells by counting the growth number of tumor cells and also by cinematography. As a result, it has been demonstrated that the regional lymph node cells from the mouse isografted with the MC-tumor (2mm³ in size) acquire a strong antitumor activity by 14 days after the transplantation, but such antitumor activity diminishes and disappears in the terminal stage of cancer. When the number of these lymphocytes is increased, there can be observed some dosage effect, but no complete inhibition of the tumor growth can be attained. The cinematographic observations of these regional lymph node cells in the mixed culture with tumor cells reveal that lymphocytes of small to intermediate size aggregate onto the tumor cells and inhibit the movement of the latter.

*PMID: 4254199 [PubMed - indexed for MEDLINE] Copyright ©OKAYAMA UNIVERSITY MEDICAL SCHOOL

Acta Med. Okayama 24, 447—456 (1970)

**IN VITRO STUDIES ON THE INHIBITORY EFFECT OF
LYMPH-NODE CELLS I. ANTITUMOR ACTIVITY OF
REGIONAL LYMPH-NODE CELLS FROM METHYLCHO-
LANTHRENE-INDUCED SARCOMA BEARING MICE ON
THE SAME PRIMARY CULTURE SARCOMA CELLS**

Masaki OHSUGI

Department of Surgery, Okayama University Medical School, Okayama, Japan
(Director: Prof. S. Tanaka)

Received for publication, June 31, 1970

With the demonstration of the presence of tumor specific antigens in cancers induced by viruses and chemical carcinogenic substances recently it has been gradually clarified that the host exhibits a specific antitumor activity against these tumor antigens by aid of lymphocytes. These are the studies mainly concerned with establishment of anti-tumor transplantability by determining tumor-take after the viable tumor cell transplantation in the hyperimmunized host with the same tumor cells. Consequently, such tumor transplantation experiments *in vivo* cannot very well be applied directly to the study of human cancers. It is, therefore, necessary to find out some experimental method that can be applied to a tumor-bearing individual destined to die soon or later if left untreated.

In animal experiments, we have already several reports demonstrating that even the mice destined to die of homotransplanted Ehrlich ascites tumor show antitumor activity in regional lymph node cells and spleen cells at an early stage of cancer as proven by mixed cultures of mouse lymphocytes and the tumor cells, and also by this *in vitro* mixed cultures such regional lymph node cells (after mixed culture with tumor cells) aggregate on the tumor cells and inhibit the growth and destroy the latter (1, 2). It is also known that even in the C3H mouse isografted with A line cells derived from C3H mouse mammary cancer, the regional lymph node cells of such a mouse clearly show antitumor activity (3).

However, in the case of Ehrlich tumor as it is a homotransplantation, it is not definite whether the host lymphocytes themselves react on tumor specific antigens, and likewise in the case of A cells, even though it be an isotransplantation, since it is a tumor cell line maintained over a long period of time, it might have undergone some mutation, hence it cannot definitely be said that the host lymphocytes react on the tumor specific

antigens of A cells.

For the purpose to clarify these problems, we studied the direct action of lymphocytes from the host mouse on primary culture tumor cells. In this study we used methylcholanthrene-induced sarcoma from C3H mouse and during isotransplanting these sarcomas to C3H mice successively for 2—3 generations observed the direct action of regional lymph node cells from these mice on the primary culture tumor cells derived from the same sarcoma. As a result it has been found that the mice receiving isotransplantation of tumor cells and destined to die do exhibit antitumor activity via lymphocytes by acting specifically on tumor-specific antigens in some early cancer stage but by the terminal stage of cancer such antitumor activity diminishes and finally disappears.

MATERIALS AND METHODS

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

Primary tumor cell culture: To 10 of C3H mice 1mg/mouse of 20-methylcholanthrene suspended in Arabian gum solution is injected subcutaneously on the back. There will be formed tumor at the site of injection 3—5 months after the injection, but the tumors grown to the size of thumb are selected and extirpated from two mice, and the tumors are sliced to the size of a rice grain and isotransplanted to C3H mice successively to serve as the test materials. Each of these tumors is designated as Ta and Tb respectively for convenience. When the tumors have grown to the size of thumb at 3—4 generations, the tumors are taken out, tumor cell suspension is prepared by the method of MADDEN-BURK (4).

The tumor thus taken out aseptically is sliced into fine pieces, to 1g each tumor piece is added 8ml cold Hanks solution+10ml 0.2% trypsin +6—7 drops of 0.04% DNase, left standing for about 2 hours, stirring every now and then, this suspension is filtered through the 80-mesh filter. To remove the cells remaining on the meshes 10ml 0.2% trypsin GKN solution and 6—7 drops of 0.04% DNase are poured, which is then stirred for about one hour with the magnetic stirrer, filtered again through the 80-mesh filter, and this filtrate is taken as H-1 (the first harvest). The tumor cells harvested from H-1 are cultured in the medium composed of YLE (Earl's balanced salt solution containing 0.1% (w/v) yeast extract and 0.5% (w/v) lactalbumin hydrolysate) supplemented with 20% calf serum. The number of viable tumor cells is determined by the eosin dye exclusion test. Taking those cells with large nucleus, distinct nucleoles and thin chromatic structure as tumor cells, the number of the cells is adjusted to 20×10^4 viable cells per ml, 1.5ml each of the sample is poured into individual short test tubes and incubated stationary at 37°C.

Sensitization of mice: C3H mice are roughly divided into 2 groups. The first group consists of 72 mice, to which 2 kinds of Ta and Tb methylcholanthrene-induced sarcomas (MC-tumor) are transplanted and the degree of antitumor activity of regional lymphocytes is observed with lapse of time. Namely, in the case using Ta the second generation transplant and with Tb, the third generation tumor, the piece measuring 2 mm³ of each is transplanted under the skin on the back between scapulas, 33, 22, 17, 13 and 8 days before extracting regional lymph nodes, with Ta; while with Tb piece it is transplanted 28, 20, 14 and 7 days before the lymph-node extraction, to make 9 subgroups. On the same day when the tissue culture is commenced, regional lymph nodes are removed from every subgroup simultaneously.

The second group consists of 10 mice, and 2 mm³ piece of Ta is transplanted on the back under the skin between scapulas just as in the case of the first group. Regional lymph nodes of these mice are removed 14 days after the transplantation when the tumor has grown to the size 1 cm³, in order to determine the dosage effect of the antitumor activity of the lymph node cells.

Lymph-node cell suspension: Axillary lymph nodes are removed aseptically from cancer-bearing C3H mice and normal C3H mice (control group). These extirpated lymph nodes are cut into small pieces with ophthalmic scissors and passed through 80-mesh filter. The filtrate is washed with cold Hanks solution by centrifugation at 2,000 rpm for 5 minutes and these washings are repeated 3 times. The final precipitate is suspended in previously described YLE medium with calf serum. We find that such a cell suspension contains over 90% viable tumor cells, and this suspension serves as the test material.

In Group 1 where fluctuations of the antitumor activity at certain intervals are observed, the cell suspension is so adjusted as to contain regional lymph node cells in the number 40 times per 1.5 ml that of the tumor cells cultured for 3 days, and in Group 2 where the dosage effect on the antitumor activity is investigated, the number of lymphocytes is adjusted to 40-fold, 100-fold, and 300-fold that of tumor cells per 1.5 ml.

Addition of lymph-node cells to the primary tumor cell culture: After culturing tumor cells for 3 day several short test tubes are picked out at random, discarding the supernatant to each test tube is added 1.5 ml crystal violet solution (containing 100 ml distilled water + 2.1 g citric acid + 50 mg crystal violet), and then incubated again at 37°C for 30 minutes. Next, the cells attached to the vessel wall are detached by gentle scraping with a rubber policeman, and by stirring a uniform cell suspension is prepared. A drop of this cell suspension is picked up in a hemocytometer, and the tumor cell counts are taken more than 6 times with each test tube to get the average number of the primary culture tumor cells. The rest of test tubes are divided into several groups each composed of 3—6 tubes, after discarding the supernatant to each test tube is added 1.5 ml of the lymphocyte suspension of the test group, and these are further incubated at 37°C for 48 hours stationary, then after discarding the supernatant crystal violet solution is added and the tumor cells attached to the vessel wall are counted.

Simultaneously, 10×10^4 Ta cells are cultured separately in the culture bottles,

TD-15 with the wall of less than 0.6mm in thickness, and to these cultured cells is added 100 volumes of the regional axillary lymphocytes obtained from the mouse 14 days after the transplantation of Ta tumor piece, and behaviors of the lymphocytes on the tumor cells are observed for 72 hours with the phase-contrast cinematography apparatus.

RESULTS

In the second generation transplantation of MC-induced tumor Ta, the tumor growth is slower than that of the second generation transplantation of Tb. That is, whereas by Ta transplantation animals begin to die of tumor around 4 weeks afterward, by Tb transplantation tumor death begins to occur within about 3 weeks.

In the Ta transplantation at 33 posttransplantation day, with exception of 6 mice having the tumors bigger than the tip of thumb, 2 animals had already died of tumor, while with the mice of the rest subgroups 4 mice died accidentally or of causes other than tumor. At this stage by selecting 3—4 mice having relatively uniform, large tumors from each of

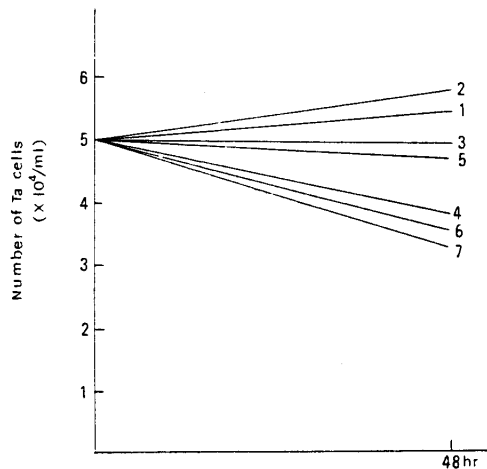


Fig. 1 Antitumor growth effect of regional lymph node cells from mice isoprotransplanted with methylcholanthrene-induced sarcoma (Ta) on primary culture Ta cells in tissue culture

1. Primary culture Ta cells alone (control)
2. Primary culture Ta cells+normal lymph node cells ($P > 0.1$)
3. Primary culture Ta cells+regional lymph node cells (8 days later) ($P > 0.05$)
4. Primary culture Ta cells+regional lymph node cells (13 days later) ($P < 0.01$)
5. Primary culture Ta cells+regional lymph node cells (17 days later) ($P < 0.05$)
6. Primary culture Ta cells+regional lymph node cells (22 days later) ($P < 0.01$)
7. Primary culture Ta cells+regional lymph node cells (33 days later) ($P < 0.01$)

these 5 subgroups, regional lymph nodes were excised, and lymph node cells were mixed with the primary culture Ta cells and cultured. The results are as shown in Fig. 1. In this instance, $20 \times 10^4/\text{ml}$ of Ta cells were placed in each culture tube, and the nuclear counts on culture day 3 amounted to $5 \times 10^4/\text{ml}$. At this stage 40-fold number of lymph node cells were added and the mixed culture was further carried out for 48 hours. It has been clearly demonstrated that the regional lymph nodes after 13 days of Ta transplantation have acquired a strong antitumor potency. It seems that a strong antitumor effect is observed in those regional lymph nodes taken out at a relatively terminal stage when the animals began to die of tumor, i. e. around 33 days after the Ta transplantation.

In the case of Tb transplantation, at 28 posttransplantation day 4 mice were already dead of tumor and only 4 survived in this subgroup, and in the subgroup of 20 posttransplantation day one mouse had died of tumor. By selecting 3 mice from each of these 3 subgroups, having the tumors of more or less the same size, their regional lymph nodes were collected, and the mixed cultures were conducted with the primary culture Tb cells. The results are as shown in Fig. 2. In those lymph node cells

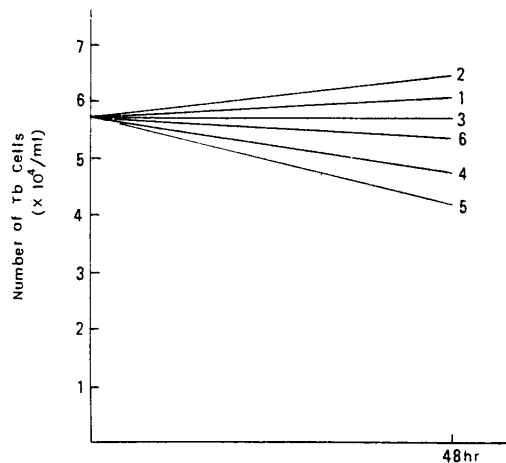


Fig. 2 Antitumor growth effect of regional lymph node cells from mice isoprotransplanted with methylcholanthrene-induced sarcoma (Tb) on primary culture Tb cells in tissue culture

1. Primary culture Tb cells alone
2. Primary culture Tb cells + normal lymph node cells ($P > 0.1$)
3. Primary culture Tb cells + regional lymph node cells (7 days later) ($P > 0.1$)
4. Primary culture Tb cells + regional lymph node cells (14 days later) ($P < 0.01$)
5. Primary culture Tb cells + regional lymph node cells (20 days later) ($P < 0.01$)
6. Primary culture Tb cells + regional lymph node cells (28 days later) ($P < 0.1$)

prepared on the 28th day of the transplantation, i. e. at the time corresponding to the terminal stage when the half of mice died, there could be observed not any antitumor activity, and there was no difference from the case of single cell culture and of the mixed culture with normal lymph node cells. However, in those lymph nodes taken out at 14 or 224 post-transplantation day there could be seen a strong antitumor activity.

In the isotransplantation of MC-induced sarcoma the regional lymph nodes around 8 posttransplantation day acquire antitumor activity. This activity gradually increases and becomes strong within about two weeks, but such antitumor activity ultimately decreases and disappears at the terminal stage.

With the second group we tried the mixed cultures of those lymph node cells with a high antitumor activity (prepared in the second posttransplantation week) in various numbers with a fixed number of Ta cells, and observed dosage effect as shown in Fig. 3. Namely, we find that 100-fold

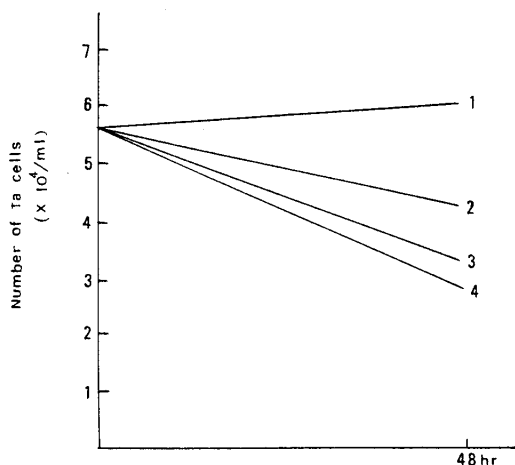


Fig. 3 Antitumor growth effect of varying doses of regional lymph node cells from mice isotransplanted with methylcholanthrene-induced sarcoma (Ta) on primary culture Ta cells in tissue culture

1. Primary culture Ta cells alone
2. Primary culture Ta cells + regional lymph node cells of 40-fold the number of Ta cells ($P < 0.01$)
3. Primary culture Ta cells + regional lymph node cells of 100-fold the number of Ta cells ($P < 0.01$)
4. Primary culture Ta cells + regional lymph node cells of 300-fold the number of Ta cells ($P < 0.01$)

and 300-fold lymph node cells show a stronger antitumor effect on Ta cells than 40-fold cells, but the difference is not so great as to be proportional

to the difference in the number of the cells added.

By the time lapse cinematography of the mixed culture of regional lymph node cells of the second posttransplantation week added in 40-fold number against Ta primary culture cells, the direct action of the lymph node cells was observed. In this instance, the pictures were taken at the rate of 1 frame per 20 seconds. Fig. 4 shows the picture taken at

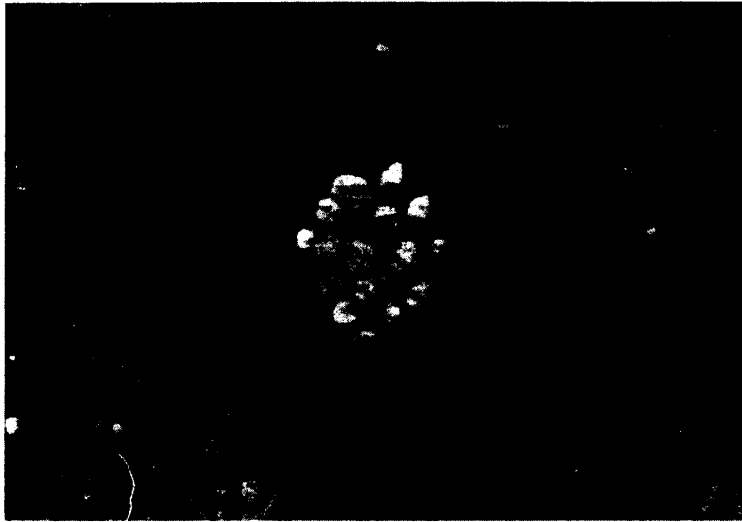


Fig. 4 Showing the mixed culture of primary culture cells with regional lymph node cells at culture hour 24
Regional lymph node cells aggregate onto the primary culture cells.

culture hour 24. Around culture hour 6 small and intermediate lymphocytes all start aggregating on the surface of the tumor cell, and with increasing number of lymphocyte aggregation, the movement of the tumor cell becomes sluggish. The tumor cell loses its projection and becomes spherical in shape, but with such a tumor cell within 36-hour observation there occurs no death from destruction nor cell division, remaining in the spherical shape with 16 lymphocytes attached on it. When the time lapses from 36 to 48 hours of culture, there can be seen many broken tumor cells, those tumor cells carrying many lymphocytes, and also many tumor cells moving actively without any attachment of lymphocytes. In the mixed culture of tumor cells where normal lymphocytes are added, no such aggregation phenomenon can be observed.

DISCUSSION

The fact that methylcholanthrene-induced tumor possesses tumor specific transplantation antigens differing from original tumor-bearing mice has been first demonstrated by the transplantation of tumor by FOLEY (5), which has been finally verified by KLEIN *et al.* (6) in their sarcoma autotransplantation experiments. In the studies on rats there are some excellent reports by the school of TAKEDA (7). These experiments, demonstrating the presence of tumor specific antigens, are all *in vivo* experiments determining "tumor-take" with animals whose tumor was ligated or extirpated or with those animal previously hyperimmunized by injecting irradiated tumor cells. When the isotransplantation of the tumor cells mixed with lymph node cells from these sensitized animals is performed to the same species of animals, "tumor-take" and the tumor growth are inhibited and the lymph node cells give the antitumor activity to the host by adoptive transfer. These facts prove that the antitumor activity is possessed by the lymphocytes of such a sensitized animal. However, such *in vivo* methods are not applicable to human subjects. It is, therefore, necessary to investigate whether or not antitumor activity is present in those tumor-bearing animals in the untreated conditions as they are without hyperimmunization.

It has already been clarified that "*in vivo* homograft reaction" is reflected in *in vitro* experiments by the mixed culture of the lymphocytes and homologous target cells of the host, and HARA (1, 2) of our laboratory has demonstrated in his homotransplantation experiments of Ehrlich ascites tumor that the lymphocytes of these tumor-bearing mice do aggregate and inhibit the growth of Ehrlich tumor cells. In the present study it has been clarified that the lymphocytes of the host receiving MC-tumor isotransplantation aggregate on the target cells and inhibit their growth. However, since the reciprocal skin graft between the C3H mice survives permanently without rejection (8), the antitumor activity of the lymphocytes of the mice transplanted with MC-tumor seems to be directed to this tumor specific antigens. The identification of lymphocytes and tumor cells in each experiment is easy from the size and shape of cells and the color tone of the nuclei. In addition, it seems that the antitumor activity of lymphocytes *in vitro* reflects the antitumor activity of the lymphocytes *in vivo*. In the neutralization experiments where the regional lymphocytes obtained from the mice one and two weeks after the isotransplantation of MC-tumor are mixed with MC-tumor cells and transplanted to the same species of mice, it has been demonstrated (9) that the "tumor-take" and the tumor

growth are inhibited. At least some of MC-tumors have tumor specific antigen and this antigen seems to resemble histocompatibility antigen.

In the isotransplantation of MC-tumor the antitumor activity of regional lymphocytes persists relatively long time even in the mice destined to die of tumor, but such antitumor activity disappears in the terminal stage of cancer. This phenomenon can be observed even in the cases of homotransplantation of Ehrlich tumor cell and isotransplantation of A cells derived from C3H mouse mammary cancer and this seems to be associated with immunological tolerance and immunological enhancement. The antitumor activity of the lymphocytes from cancer-bearing mouse is of a relative nature, as such even when lymphocytes in number 100 times or 300 times that of tumor cells are cultured with the tumor cells, a complete inhibition of the proliferation of the tumor cells cannot be attained. Admitting that the antitumor activity in the cancer-bearing body is not so strong as to completely inhibit its own cancer cells, if some means to increase such antitumor activity can be developed, it would be of a great use in cancer treatment.

SUMMARY

As a step in the elucidation of immunity of human cancer from the standpoint of homotransplantation immunity, we conducted mixed cultures of regional lymph node cells from C3H mouse isotransplanted with methylcholanthrene-induced sarcoma (MC-tumor) together with the primary culture MC-tumor cells, and observed the behaviors of these lymph node cells to the MC-tumor cells and compared the effects of these lymph node cells with those of normal mouse lymph node cells by counting the growth number of tumor cells and also by cinematography. As a result, it has been demonstrated that the regional lymph node cells from the mouse isotransplanted with the MC-tumor (2mm³ in size) acquire a strong antitumor activity by 14 days after the transplantation, but such antitumor activity diminishes and disappears in the terminal stage of cancer.

When the number of these lymphocytes is increased, there can be observed some dosage effect, but no complete inhibition of the tumor growth can be attained.

The cinematographic observations of these regional lymph node cells in the mixed culture with tumor cells reveal that lymphocytes of small to intermediate size aggregate onto the tumor cells and inhibit the movement of the latter.

ACKNOWLEDGEMENT

The author expresses profound thanks to Prof. SANAÉ TANAKA and Dr. KUNZO ORITA for painstaking proof-reading and kind guidance throughout the present experiments.

REFERENCES

1. HARA, S.: Cellular antibody in mice bearing Ehrlich cancer. I. A quantitative study on antitumor activity of cellular antibody *in vitro*. *Acta Med. Okayama* **19**, 91, 1965
2. HARA, S.: Cellular antibody in mice bearing Ehrlich cancer. II. Properties of lymphoid cells from sensitized animal. *Acta Med. Okayama* **19**, 99, 1965
3. SATOH, K.: *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. *Acta Med. Okayama* **20**, 261, 1966
4. MADDEN, R. E. and BURK, D.: Production of viable single cell suspensions from solid tumors. *J. Nat. Cancer Inst.* **27**, 841, 1961
5. FOLEY, E. J.: Antigenic properties of methylcholanthrene-induced tumors in mice of the strain of origin. *Cancer Res.* **13**, 835, 1953
6. KLEIN, G., SJÖGREN, H. O., KLEIN, E. & HELLSTRÖM, K. E.: Demonstration of resistance against methylcholanthrene-induced sarcomas in the primary autochthonous host. *Cancer Res.* **20**, 1561, 1965
7. TAKEDA, K., KIKUCHI, Y., YAMAWAKI, S., UEDA, T. and YOSHIKI, T.: Treatment of artificial metastasis of methylcholanthrene-induced rat sarcomas by autoimmunization of the autochthonous hosts. *Cancer Res.* **28**, 2149, 1968
8. KOKUMAI, Y.: Studies on the reaction of host lymphoid system in homotransplantation. I. Humoral and cellular reactions against the transplanted cells. *Acta Med. Okayama* **20**, 235, 1966
9. KASHIHARA, E.: Inhibitory effect of isologous regional lymph node cells from the methylcholanthrene-induced sarcoma bearing mice on the tumor *in vivo*. *Acta Med. Okayama* **24**, 161, 1969