Bull. Inst. Chem. Res., Kyoto Univ., Vol. 47, No. 1, 1969

Studies on Transplantation Immunity of Methylcholanthrene-Induced Tumor in Mice

Morihisa KITANO*

Received January 13, 1969

For the purpose of investigating whether transplanted tumor grafts in mice immunized by the same tumor are destroyed by cellular antibodies, by humoral antibodies, or by both, MC-induced sarcomas contained in single or double diffusion chambers were implanted intraperitoneally in isologous mice sensitized with MC-sarcoma irradiated with ^{60}Co.

The single diffusion chamber method in mice sensitized by the same tumor showed a remarkable immune resistance to target tumors in both cell-permeable and cell-impermeable diffusion chambers, whereas no resistance was observed in mice sensitized by normal tissue. The double diffusion chamber method with cell-permeable MF used as the septal membrane, showed again that injury of the target tumor seemed to be due to contact with sensitized lymphocytes, but when cell-impermeable MF was used as the septal membrane target cell injury occurred even without direct contact with sensitized lymphocytes. Therefore, it can be concluded that when lymphocytes can pass through the membrane of the diffusion chamber, target tumor cells are destroyed by contact with sensitized lymphocytes, but when they cannot pass through the membrane, sensitized lymphocytes go as close as possible to the target cells and a humoral substance from the lymphocytes plays a large role in the immune response.

INTRODUCTION

In 1943, Gross[11] showed that methylcholanthrene-induced sarcomas of C3H mice can induced resistance in their hosts against transplantation of the same tumor originated from isologous mice. It has been confirmed by Foley[8], Prehn[24] and Révész[27], and analogous results have been obtained by Klein on methylcholanthrene-induced sarcomas in autochthonous mice. Some investigators[1,20,23,36] later reported the successful induction of resistance to the growth of transplanted mouse tumors by pretreatment with x-ray irradiated cells from the same kind of tumors.

According to these investigations it seems to be proved that the hosts acquire an immunological resistance to their own tumors and tumors of isologous origin. In the previous reports[14,16], the author showed that transplantation immunity of Ehrlich ascites carcinoma can be induced by sensitizing with the same kind of tumors generally or locally. Since then, I attempted to confirm the same results of MC-induced sarcoma, to reveal difference of the immunological response between the regional lymph node and other parts. Therefore, the question arose as to whether the resistance against tumor transplantation in the immunized

* 北野 司久: Department of Thoracic Surgery (Head: Prof. Chuzo Nagaishi, M. D.), Chest Disease Research Institute, Kyoto University, Kyoto.

host was formed through cellular or humoral factors. An experimental approach to this problem became possible through the availability of porous filters, *i. e.* diffusion chamber, which would permit the passage of essential metabolities, but would prevent the passage of the sensitized host.

Since 1954 many reports[3,7,9] have been published on the diffusion chamber, a technic for culturing cells *in vivo*. Those chambers did not permit quantitative measurement of cells grown in them, and were too large to use for experiments in mice. Thererfore, the author attempted to design a favourable diffusion chamber for experiments in mice.

In this paper, the author[15] comfirm that transplantation immunity of MC sarcoma can be induced by pretreatment with the same tumor generally or locally, and try to analyze which antibody, cellular or humoral, chiefly acts on transplanted tumor cells.

MATERIAL AND METHODS

Animals: The animals used in this study were 2~3 month old DDD-s mice, weighing 20~25 gm. This strain was maintained by continuous, single line brother to sister mating at the Inbred Strain Animal Center at Kyoto University. All mice were kept on a standard pellet diet which, together with drinking water, was available *ad libitum*.

Tumor: Sarcomas were induced by subcutaneous injection of 0.5 to 1.0 mg methylcholanthrene (L. Light & Co., Ltd in England) dissolved in 0.1 ml olive oil into the dorsal skin of mice, and they were maintained by subcutaneous transplantation at two to three week intervals. As the antigenicity of tumors may be changed by many passages, tumors transplanted for more than five generations were not employed in the present experiments.

Some of these tumors were inoculated subcutaneously into untreated isologous mice to maintain the tumor, while the rest were irradiated with a total dose of 15,000 R of ^{60}Co (30 R/sec) mixed with an equal volume of physiolgical saline containing 100 I.U. penicillin and homogenized in a Virtis blender at approximately 3,000 r.p.m. for 5 minutes; the tumor homogenate was used as the antigen. The homogenates were kept frozen at −20°C. The control fluid injected on the same day consisted of pooled liver, kidney, and spleen tissues of untreated isologous mice or of physiological saline. The tumor cells employed as target cells in the diffusion chamber were usually in the form of tumor about 2 mm^3 in size.

Diffusion Chamber: The author adopted the diffusion chamber designed by Algire[3] and Gabourel[10] so that it could be used for experiments with mice. The acryloid ring shown in Fig. 1 has an outside diameter of 10 mm and an inside diameter of 8 mm, ring B is 4.5 mm thick and ring A is 1.5 mm thick. The Millipore Filter (MF) used in these experiments were cell-permeable AA filters or cell-impermeable HA filters.

The method devised by the authors, as illustrated in Fig. 2, will be called the diffusion chamber method of the Chest Disease Research Institute of Kyoto

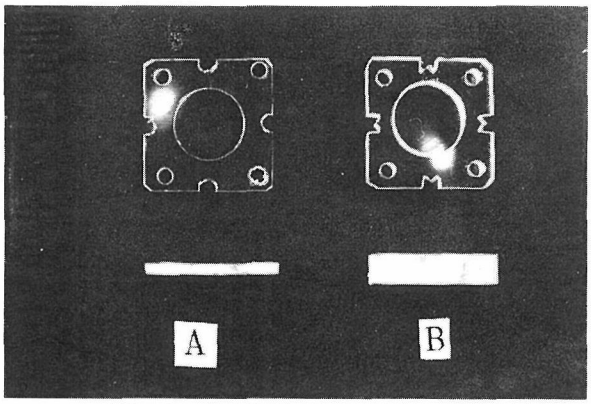

Fig. 1. Acryloid rings.
A : Ring A B : Ring B.

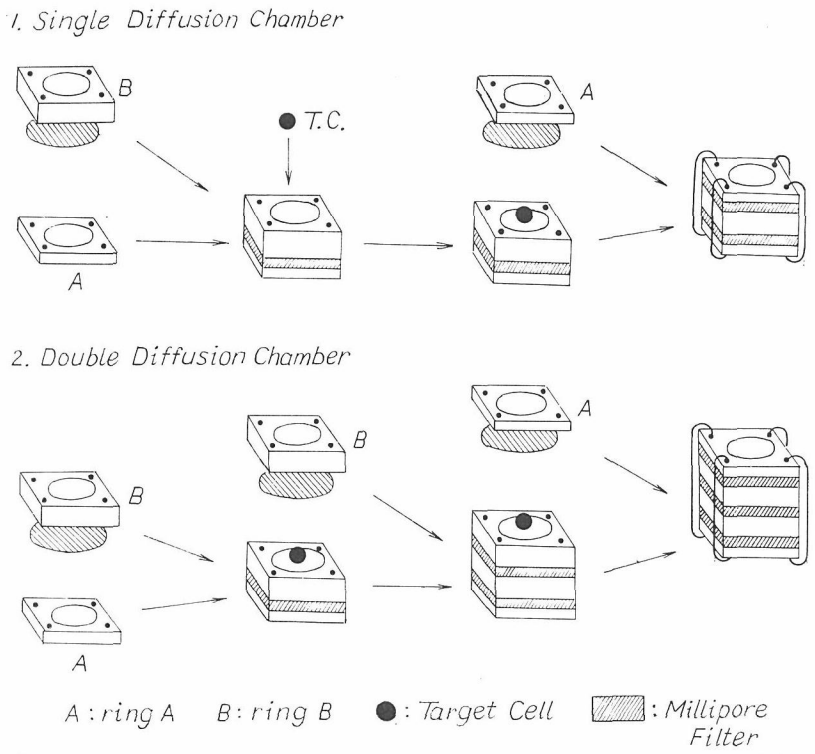

Fig. 2. Diffusion chamber method of the Chest Disease Research Institute, Kyoto University (Kyodai-Kyobuken Ho in Japanese).

University (Kyodai-Kyobuken Ho in Japanese). The acryloid rings were sterilized by being steeped in 70% alcohol or Hibitan Digluconate for 30 minutes, then washed three times in physiological saline. The MF were placed in sterilized flasks and exposed to ultraviolet light for one hour.

The procedure for assembling the diffusion chamber is illustrated in Fig. 2. An MF is inserted between an ring A and B to form a small dish. The adhesive

used for sealing is a 1% solution of MF in acetone. After confirmation of the watertightness of the dish by filling it with Hanks solution, the target cells were placed in the center of the dish. Then the dish was covered with another MF and ring A to biuld a small chamber filled with Hanks solution and target cells. The four corners of the chamber were tied tightly with silk threads. This chamber is called a Single Diffusion Chamber and is diagrammed in Figs. 3 and 4. Double Diffusion Chambers were also constructed, as illustrated in Figs. 3 and 4. These chambers were inserted into the peritoneal cavities of mice anesthetized by the intraperitoneal injection of 1.5~2.0 mg of nembutal sodium.

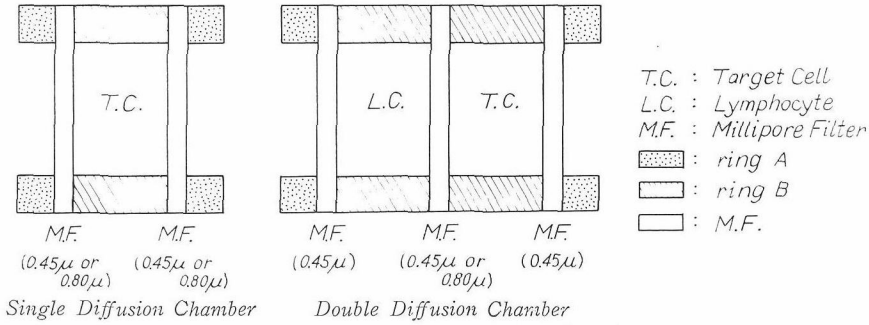

Fig. 3. Construction of Diffusion Chambers.

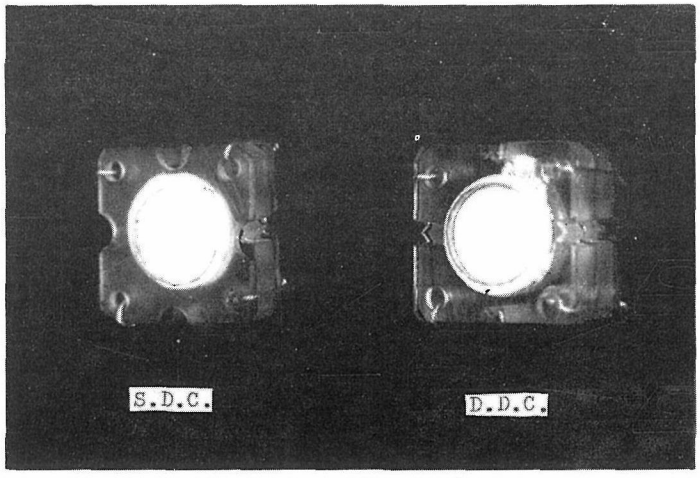

Fig. 4. Assemblied chambers.
S.D.C. : single diffusion chamber D.D.C. : double diffusion chamber

Method of Immunization: When general immunization was intended, 0.15~0.2 ml of tumor homogenate was injected into the subcutaneous tissue and peritoneal cavity. For local immunization, a total dose of 0.05~0.10 ml of tumor homogenate was injected into the thigh and the foot pad. These injections were given once a week for four weeks.

For BCG sensitization, 0.5 mg of BCG with complete adjuvant was injected into the dorsal skin of DDD-s mice, and one week later 0.5 mg of BCG was injected intravenously. Two weeks later, sensitization was confirmed by the

Mantoux test.

In transplantations to the dorsal skin of mice, tumor cells were inoculated subcutaneously without anesthesia. In transplantations to the lymph nodes, tumor cells were inoculated with a tuberculin syringe directly into a lymph node exposed by skin incision under general anesthesia. Diffusion chambers were inserted into the peritoneal cavity under general anesthesia.

Usually on the 28th day after tumor transplantation, the animals were killed, and any tumors found were removed and weighed.

On the 28th day after the insertion of a diffusion chambers, the animal was killed and the chamber removed. The degree of tumor growth was estimated by the size of the tumor mass in the diffusion chamber and classified into six categories as follows (Fig. 5) :

(−).....no tumor in the diffusion chamber.
(+).....tumor mass occupying 1/5 of the diffusion chamber.
(++).....tumor mass occupying 2/5 of the diffusion chamber.
(+++).....tumor mass occupying 3/5 of the diffusion chamber.
(++++).....tumor mass occupying 4/5 of the diffusion chamber.
(++++).....tumor mass filling the diffusion chamber completely.

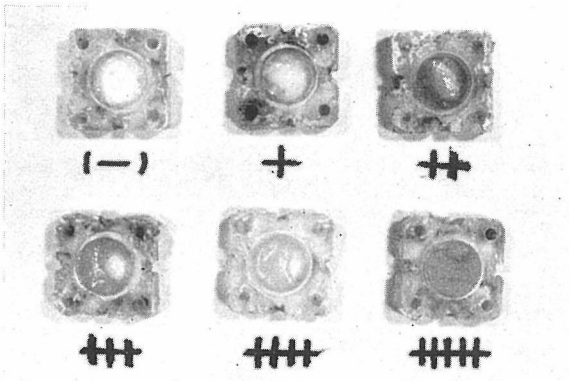

Fig. 5. Classification into six categories by the size of target tumor mass in the diffusion chamber.

For histologic examination the removed tumors and the chambers containing tumor tissue were fixed in 10% formalin ; then the acryloid ring was dissolved in chloroform. The tumor tissue was embedded in paraffin, cut in sections, and stained with hemtoxylin and eosin.

RESULTS

A. Effects of General Immunization on Transplanted Methylcholanthrene-induced Tumors

1. Transplantation to subcutaneous tissue

A thick mixture containing $10^6 \sim 10^7$ methylcholanthrene-induced tumor cells was transplanted into the dorsal skin of three groups of mice. The first group

had been immunized by subcutaneous and intraperitoneal injections of irradiated methylcholanthrene tumor homogenate; the second group had been injected with physiological saline; and the third group was untreated. Transplanted MC tumor cells were rejcted by 60% of immunized mice, 40% of the saline injected and by

Table 1. Effect of immunization on transplantation of MC-induced sarcomas in the dorsal skin.

Mouse No.	(I) Group immunized with ^{60}Co-irradiated MC-tumor	(II) Control group treated with physiological saline	(III) Control group untreated
	Occurrence of tumor	*Occurrence of tumor*	*Occurrence of tumor*
#1	0.7	3.3	4.8
2	0.15	2.9	4.4
3	0.1	1.7	2.9
4	0.1	0.6	2.1
5	(−)	0.15	0.6
6	(−)	0.1	0.2
7	(−)	(−)	(−)
8	(−)	(−)	(−)
9	(−)	(−)	(−)
10	(−)	(−)	(−)
Rate of "takes"	*4/10*	*6/10*	*6/10*
Average weight	0.26 gm	1.44 gm	2.50 gm

Table 2. Effect of immunization on transplantation of MC-induced sarcomas in the dorsal skin.

Mouse No.	(I) Group immunized with ^{60}Co-irradiated MC-tumor	(II) Control group immunized with ^{60}Co-irradiated normal tissue	(III) Control group treated with physiological saline
	Occurrence of tumor	Occurrence of tumor	Occurrence of tumor
#1	(−)	(−)	(−)
2	(−)	(−)	(−)
3	(−)	0.10	(−)
4	(−)	0.35	0.20
5	0.25	0.45	0.65
6	0.30	1.66	2.22
7	0.32	2.10	3.20
8	0.36	3.32	3.54
9	0.40	3.58	3.76
10	0.90	3.66	4.10
11	0.92	3.72	4.32
12	1.51	4.30	4.64
13	1.98	4.70	4.82
14	2.20	6.25	5.68
15	3.30	6.04	
Rate of "takes"	11/15	13/15	11/14
Average weight	1.11 gm	3.02 gm	3.38 gm

40% of the untreated mice. The weight of the tumors averaged 0.26 gm in the immunized mice, and 1.44 gm and 2.50 gm in the controls as shown in Table 1.

The thick mixture of MC tumor cells was also transplanted in three other groups: one which had been immunized by the same tumor tissue, one immunized by normal tissue (liver, spleen and kidney) and one which had been injected with physiological saline. As shown in Table 2, the average weight of the removed tumors was 1.11 gm in the first group, 3.02 gm in the second and 3.38 gm in the third group. These results revealed that micesensitzed by MC-induced sarcoma were resistant to transplantation of the same tumor.

2. Transplantation into lymph nodes

MC tumor cells were inoculated into the dorsal skin lymph nodes, and the growth-rates of transplanted tumors in mice sensitized with the same tumor were compared with those of control mice. MC tumors transplanted into lymph nodes appeared to grow more rapidly than those transplanted into subcutaneous tissue. However, the growth of tumors transplanted into the lymph nodes of immunized mice was slower than that in non-immunized mice.

Table 3. Effect of general immunization on transplantation of MC-induced sarcomas into inguinal nodes.

Mouse No.	Immunized group		Control group	
	r-inguinal lymph node	l-inguinal lymph node	r-inguinal lymph node	l-inguinal lymph node
#1	2.5 gm	2.0 gm	6.7 gm	4.1 gm
2	1.8	3.1	4.4	3.9
3	1.6	1.8	3.3	5.5
4	0.6	3.9	2.3	5.4
5	0.5	1.7	2.6	3.1
6	0.8	1.3	2.3	3.1
7	0.2	1.3	1.8	2.3
8	0.5	0.8		
9	0.6	0.4		
Average weight	1.41 gm		3.62 gm	

The results are shown in Table 3. The average weight of the tumors was 1.41 gm in mice immunized by tumor tissue, and 3.62 gm in control mice.

MC sarcoma cells were transplanted into the lymph nodes of three groups of mice. The first group was immunized by irradiated MC sarcoma, the second group was sensitized with BCG and third group was untreated. As is shown in Table 4, transplanted MC sarcomas were rejected in 38% of mice immunized by tumor, in 6% of those sensitized with BCG, and in 6% of untreated mice. The average weight of the tumors was 0.63 gm in the first group, and 0.65 gm and 1.28 gm in the control groups.

Histologically, transplanted tumor tissues were seen in the central part of the lymph node invading surrounding lymphoid tissue as shown in Fig. 6. In the central portion of transplanted tumor tissues, cells frequently were found to

Table 4. Effect of general immunization on transplantation of MC-induced sarcomas into inguinal nodes.

Experimental groups	Lymph nodes	Case No. of mice									Rate of "takes"	Average weights
		#1	2	3	4	5	6	7	8	9		
I	right	(−)	(−)	(−)	0.65	0.60	0.65	0.45	1.10		10/16 (62.5%)	0.63 gm
	left	(−)	(−)	0.55	(−)	0.35	0.60	0.65	0.70			
II	right	(−)	0.15	0.20	0.20	0.60	0.65	0.65	1.35	1.90	17/18 (94.4%)	0.65 gm
	left	0.75	0.25	0.45	0.45	0.55	0.55	0.75	0.95	0.60		
III	right	1.40	0.50	0.80	1.30	1.40	1.55	1.40	2.20		15/16 (93.7%)	1.28 gm
	left	(−)	0.45	1.45	1.25	0.90	1.10	1.35	2.00			

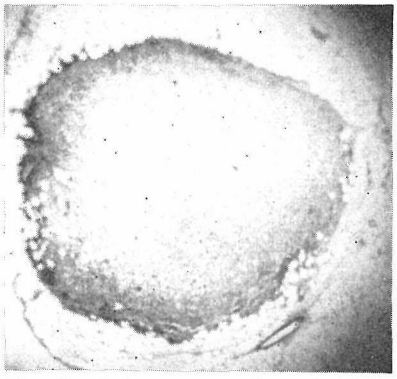

Fig. 6. A low power view of transplanted MC tumor in lymph nodes of generally immunized mice. Central-necrosis of tumor tissue is seen. (H.-E. staining)

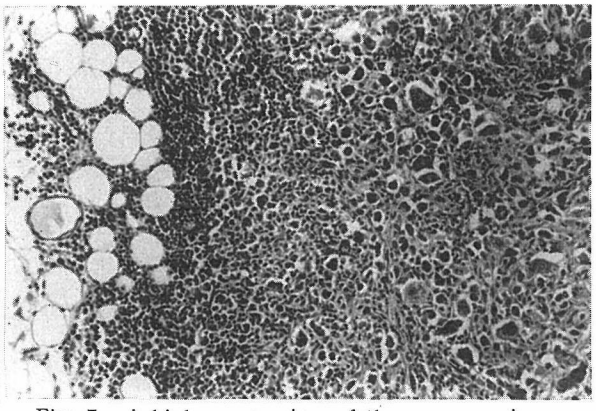

Fig. 7. A high power view of the same specimen in Fig. 6. Marked lymphocyte-infiltration and giantic tumor cells are found. (H.-E. staining)

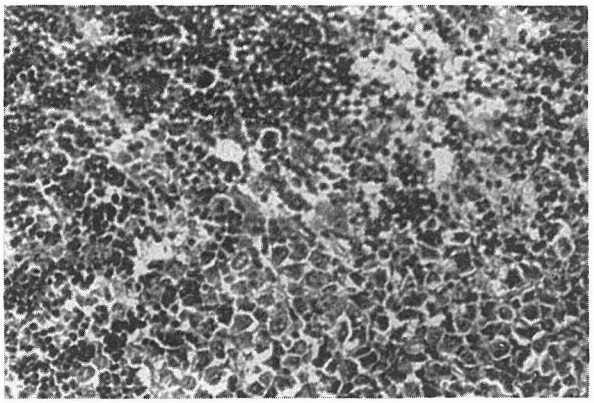

Fig. 8. Transplanted tumor in lymph nodes of generally immunized mice. Marked lymphocyte-infiltration in tumor tissues can be seen. (H.-E. staining)

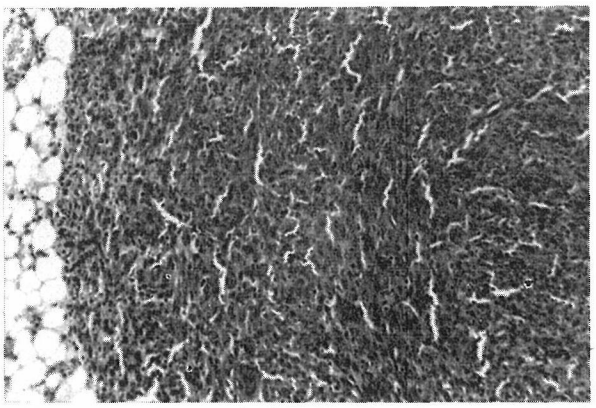

Fig. 9. Transplanted tumor in lymph nodes of non-immunized mice. Tumor cells only can be seen. (H.-E. staining)

be degenerated, while in the peripheral zone of the tumor tissue, most cells were vigorous and infiltrating the adjacent lymphoid tissue, as shown in Fig. 7. Immunized animals, the peripheral parts of the tumor tissu were seen to be divided into small groups by lymphocyte-infiltration, as shown in Fig. 8. On the contrary, transplantation tumor tissues only were seen in the lymph nodes of control mice, and lymphocytes were very scarce as shown in Fig. 9.

These facts indicate that immunological resistance to the transplantation of MC sarcoma can generally be demonstrated in the subcutaneous tissue and lymph nodes of mice sensitized with the same tissue.

B. Effects of Local Immunization on Transplanted Methylcholanthrene-induced Tumors

This experiment was designed to demonstrate the difference in transplantation immunity between sensitized and unsensitized regional lymph nodes. After local immunization with MC sarcoma tissue, 0.0125 ml of tissue mixture of the same tumor was injected into immunized and into non-immunized lymph nodes, and the rates of "takes" and growth of transplanted tumors were observed for four weeks.

The results showed no significant difference in the rate of successful "takes" of transplanted tumors between the local by immunized nodes and the controls,

Table 5. Effect of local immunization with ^{60}Co-irradiated MC-induced sarcomas on transplantation of the same tumor into regional lymph node.

	No. Mouse	#1	2	3	4	5	6	7	8	9	10	Rate of "takes"	Average weights
Immunized inguinal lymph nodes	Occurrence of tumors	(−)	(−)	(−)	(−)	(+)	(+)	(+)	(+)	(+)	(+)	6/10	1.60 gm
	Weight of tumors					0.75	1.0	1.2	1.4	1.9	3.35		
Non-immunized inguinal lymph nodes	Occurrence of tumors	(−)	(−)	(−)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	7/10	2.68 gm
	Weight of tumors				0.95	1.6	2.3	2.8	3.2	3.8	4.1		

as shown in Table 5. However, the average weight of the tumors was 1.60 gm in the inguinal lymph nodes on the immunized side, and 2.68 gm on the non-immunized side.

It can be concluded that the antibody related to transplantation immunity of tumor tissue is not equally distributed through out body, but concentrated in in the regional lymph nodes draining the side sensitized by ^{60}Co-irradiated tumor cells.

C. Effects of Immunized Lymphoid Cells on MC-induced Sarcoma Transplanted to Subcutaneous Tissue

Mixtures of MC tumor cells (2.3×10^5) and immunized or non-immunized lymphoid cells (4.23×10^6 or 4.23×10^5) were transplanted into the dorsal subcutaneous tissue of non-treated mice, and the mice were sacrificed four weeks later. Table 6 shows that transplanted tumors grew more rapidly in the control group than in the immunized group. The take-rate of transplanted tumors was higher in the control group than in the immunized group. The inhibition-effet of lymphoid cells against tumor cells was observed in cases where the number of lymphoid cells considerably surpassed that of tumor cells. The inhibition-effect seemed to be stronger in the lymphoid cells of the spleen than of the lymph nodes. The average weight of the tumors grown in the immunized groups was much lower than in the control groups.

Table 6. Subcutaneous transplantation of MC-induced sarcomas mixed with sensitized lymphocytes.

	Source of lymphocytes	Number of lymphocytes	Number of tumor cells	"Take" rates of transplanted tumors	Average weight (gm)
Immunized group	L	4.23×10^6	2.3×10^5	2/6	0.47
	L	4.23×10^5	2.3×10^5	7/7	1.22
	S	4.23×10^6	2.3×10^5	1/7	0.30
	S	4.23×10^5	2.3×10^5	5/7	0.84
Control group	L	4.23×10^6	2.3×10^5	6/7	1.46
	L	4.23×10^5	2.3×10^5	7/7	1.64
	S	4.23×10^6	2.3×10^5	6/7	1.98
	S	4.23×10^5	2.3×10^5	6/7	1.82
	Physiological saline		2.3×10^5	7/7	1.92

L : lymph node S : spleen

These results show that lymphoid cells from animals immunized by MC-induced sarcoma could inhibit *in vivo* the growth of transplanted cells of the same tumor.

D. Effects of General Immunization on MC-induced Sarcoma in the Diffusion Chamber

1. Insertion into the pritoneal cavity of cell-permeable diffusion chamber containing MC-induced sarcoma

A type AA Millipore Filter with pores 0.80μ in diameter was use in the cell-

permeable diffusion chamber. On the 28th day after the insertion of a diffusion chamber containing the MC-induced sarcoma, both immunized and control mice were killed and the size of tumor tissue in the chamber was determined. The data are summarized in Table 7. The growth of the target cells averaged +～ ++ in the immunized group, and +++ in the control group; that is, tumor growth was significantly suppressed in the immunized group.

Table 7. Growth of MC-induced sarcoma cells in cell-permeable diffusion chamber implanted into peritoneal cavity of mice.

Mouse No.	#1	2	3	4	5	6	7	8	Average
Immunized group	(−)	(−)	+	+	++	+++	+++	+++	+ ～ ++
Control group	+	++	+++	+++	++++	+++	+++	++++	+++

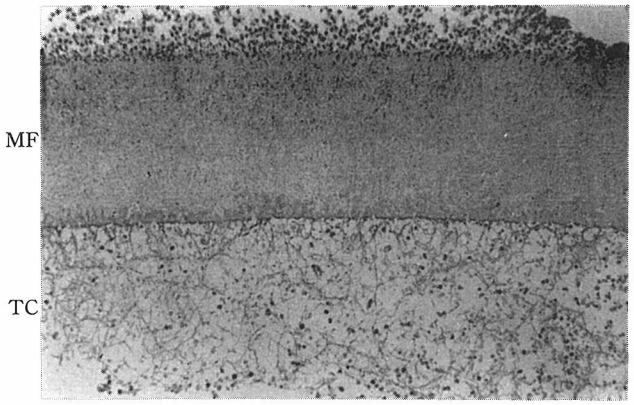

Fig. 10. Photomicrograph of cross section through single diffusion chamber removed from the peritoneal cavity of an immunized mouse. Most of MC tumor cells were suffered from cytolysis. (H.-E. staining)
MF: millipore filter, TC: target cells.

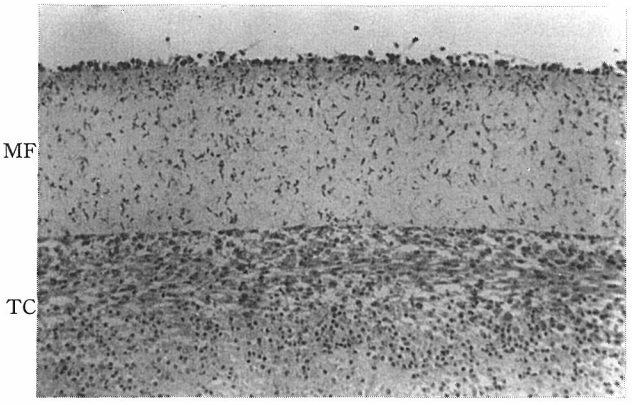

Fig. 11. Photomicrograph of cross section through single diffusion chamber removed from the peritoneal cavity of a control mouse. The central necrosis of target tumor tissue due to rapid growth was also observed, while the tumor tissue in the vicinity of the filter was lively. (H.-E. staining)

Histological examination showed that many cells of the host had migrated through the MF (Figs. 10 and 11).

In mice immunized by MC-induced tumor cells, the target cells in the chamber grew and formed a mass in the early stage, then gradually degenerated as lymphocytes infiltrated into the chamber. Large accumulations of host lymphocytes were also seen on the outer side of the MF. In the degenerated area, the arrangement of tumor cells was loose and irregular. Most of the tumor cells in the chamber should cytolysis, and only a few living tumor cells remained, as shown in Fig. 10. In the vicinity of the MF, tumor tissue was markedly degenerated and naked nuclei due to cytolysis of tumor cells were abundantly seen.

On the contrary, in the control group tumor cells were closely packed together, and lymphocytes, erythrocytes and polynuclear leukocytes had migrated through the AA MF, though the number on both sides of the MF was much lower than in the immunized group. In the control group, living tumor cells usually occupied the whole diffusion chamber. In this group, central necrosis of the target tumor tissue due to rapid growth was often observed, while the tumor tissue in the vicinity of the filter was alive, as shown in Fig. 11.

These findings indicate that the lymphocytes of the host play a large role in the immunological resistance to transplanted MC-induced sarcoma in the diffusion chamber.

2. Insertion into the peritoneal cavities of mice of cell-impermeable diffusion chambers containing MC-induce sarcoma

Type HA Millipore Filters with pores 0.45μ in diameter were used in the cell-impermeable diffusion chambers. This type of MF does not allow host cells but only fluid to enter the chamber so that one can distinguish between cellular and humoral mechanisms in the immunity to tumor transplantation. On the 28th day after the insertion of the chamber into the peritoneal cavity both immunized and control mice were killed and the tumor tissue growing in the diffusion chamber was examined.

Table 8. Growth of MC-induced sarcoma cells in cell-impermeable diffusion chamber implanted into peritoneal cavity of mice.

Mouse No.	#1	2	3	4	5	6	7	8	Average
Immunized group	(−)	(−)	(−)	(−)	(−)	(−)	(−)	(−)	(−)
Control group	+	+	+	++	+++	+++	++++	++++	+++

In the immunized group, none of the transplanted tumors in the chambers grew, while in the control group all transplanted tumors grew, the average growth being +++ as shown in Table 8. In the immunized animals, most of the target cells in the chamber were necrotic and a large number of lymphocytes and a few erythrocytes and polynuclear leucocytes were found on the outer surface of the MF, as shown in Fig. 12. No migration of host cells through the

filter was ever seen. In one animal that appeared to be highly sensitized to tumor tissue, the target tumor tissu in the chamber had degenerated completely and many lymphocytes had been attracted to the surface of the filter.

In the control group, there were far fewer lymphocytes on the surface of the filter than in the immunized group, as shown in Fig. 13.

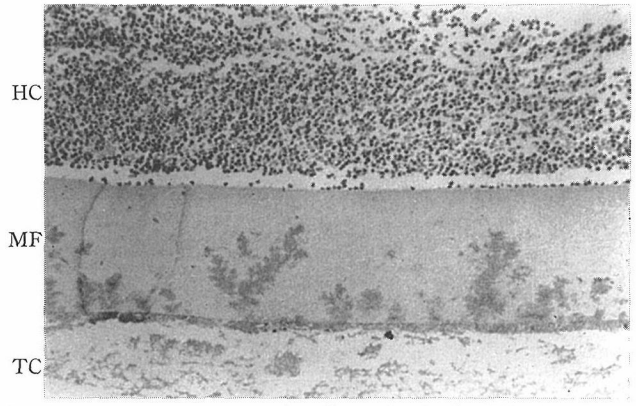

Fig. 12. Photomicrograph of cross section through single diffusion chamber removed from the peritoneal cavity of an immunized mouse. A large number of host cells (HC) was attracted to the surface of the filter. (H.-E. staining) HC : host cells, MF : millipore filter, TC : target cells.

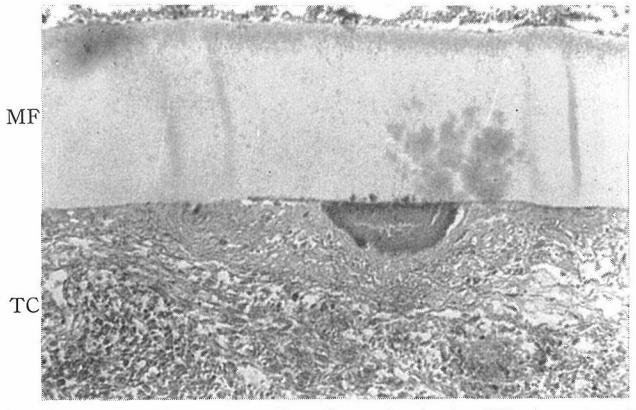

Fig. 13. Photomicrograph of cross section through single diffusion chamber removed from the peritoneal cavity of a control mouse. (H.-E. staining)

This experiment revealed again that lymphocytes play a large role in the immunological suppression of tumor growth. However, the humoral factor cannot be ignored, since the growth of transplanted tumors was markedly suppressed in cell-impermeable chambers.

E. Effect of Immunized Lymphoid Cells on MC-induced Sarcoma in Double Diffusion Chambers

1. Intraperitoneal insertion of the double diffusion chambers with a cell-permeable millipore filter as the septal membrane

Type AA Millipore Filters with pores 0.80μ in diameter were used as septal

membranes in double diffusion chambers, and type HA MF with pores 0.45μ in diameter as outside membranes, as shown in Fig. 3. In principle this double diffusion chamber does not allow host cells but only fluid to enter the chamber. Moreover, host cells could not act the target cells in the double diffusion chamber, because all the mice employed in this experiment were irradiated twice with sublethal doses (350R) of ^{60}Co so that reticulo-endothelial function was strongly suppressed. Into one chamber of the double diffusion chamber was placed target MC tumor cells and into the other immunized lymphoid cells. In this way one can expect to analyse the immunological mechanisms, especially the cellular factor, acting tumor transplantation. On the 28th day after the insertion of the double diffusion chamber containing the MC-induced sarcoma plus lymphoid cells, both experimental and control mice were killed and the tumor tissue growing in the chambers was examined. The results are summarized in Table 9.

Table 9. Growth of MC-induced sarcoma cells in double diffusion chamber with cell-permeable millipore filter as septal membrane.

Mouse No.	#1	2	3	4	5	6	7	Average
Experimental group sensitized lymphocytes	+	++	+++	+++	++++	++++	++++	+++
Control group non-sensitized lymphocytes	++++	++++	++++	++++	++++	++++	++++	++++

The average growth of target cells was +++ in the experimental group with sensitized lymphocytes, and ++++ in the control group with non-sensitized lymphocytes.

Histological studies showed that may sensitized lymphocytes had migrated through the septal membrane of MF and clustered around the tumor cells, as shown in Fig. 14. The growth of target tumor cells in the double diffusion

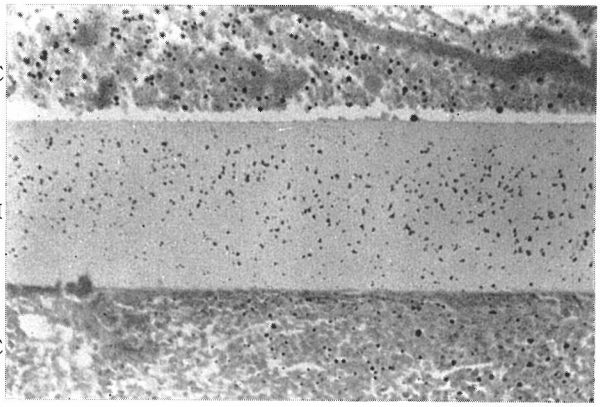

Fig. 14. Photomicrograph of cross section through double diffusion chamber containing the MC tumor plus sensitized lymphoid cells. A large number of lymphocytes migrated through the septal membrane and clustered around the tumor cells. (H.-E. staining)
TC: target cells, LC: lymphoid cells, SM: septal membrane of MF.

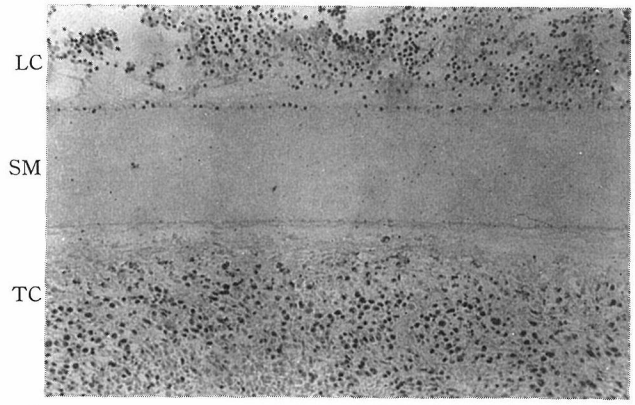

Fig. 15. Photomicrograph of cross section through double diffusion chamber containing the MC tumor plus non-sensitized lymphoid cells. Almost no lymphocytes migrated through the septal membrane. (H.-E. staining)

chamber thus seemed to be suppressed immunologically by the sensitized lymphocytes. On the contrary, in the control group with non-sensitized lymphocytes, almost no lymphocytes migrated through the septal membrane and the growth of target tumor cells was not suppressed, as is shown in Fig. 15.

These findings indicate that sensitized lymphocytes must play a large role in the immunological resistance to the growth of MC tumor cells in double diffusion chambers.

2. Intraperitoneal insertion of double diffusion chambers with cell-impermeable millipore filter as septal membrane

Type HA Millipore Filters with pores 0.45μ in diameter were used for both septal and outside membranes, as shown in Fig. 3. Host animals were irradiated twice with sublethal doses (350R) of ^{60}Co as in the above experiment. Neither sensitized lymphoid cells nor host cells can pass through the MF membranes into the chamber. By this method one can expect to analyse the immunological mechanisms, especially the humoral factors, in tumor transplantation. On the 28th day after the insertion of double diffusion chambers containing MC-induced sarcoma and lymphoid cells, both experimental and control mice were killed, and the tumor tissue growing in the chamber was examined.

As shown in Table 10, the average growth of the target cells was ++ in the experimental group with sensitized lymphocytes and +++ in the control group with non-sensitized lymphocytes. Thus, even when cell-impermeable MF is used as the septal membrane the growth of target tumor cells is suppressed considerably.

Table 10. Growth of MC-induced sarcoma cells in double diffusion chamber with cell-impermeable millipore filter as septal membrane.

Mouse No.	#1	2	3	4	5	6	7	Average
Experimental group sensitized lymphocytes	(−)	+	+	++	++	+++	+++	++
Control group non-sensitized lymphocytes	(−)	+	+	++	+++	+++	+++	+++

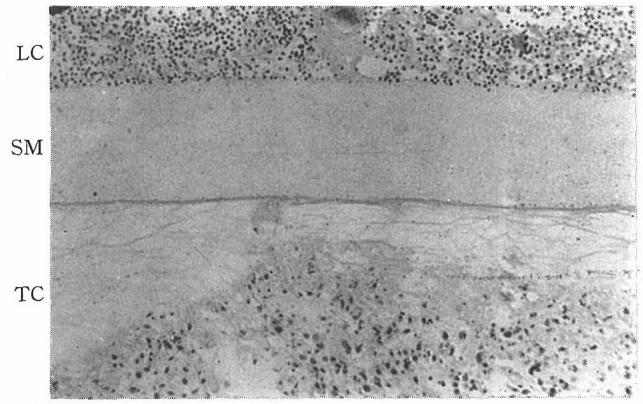

Fig. 16. Photomicrograph of cross section through double diffusion chamber containing the MC tumor plus sensitized lymphoid cells. Lymphoid cells never migrated through the septal membrane, while the tumor tissu in the vicinity of septal membrane was suffered from cytolysis. (H.-E. staining)

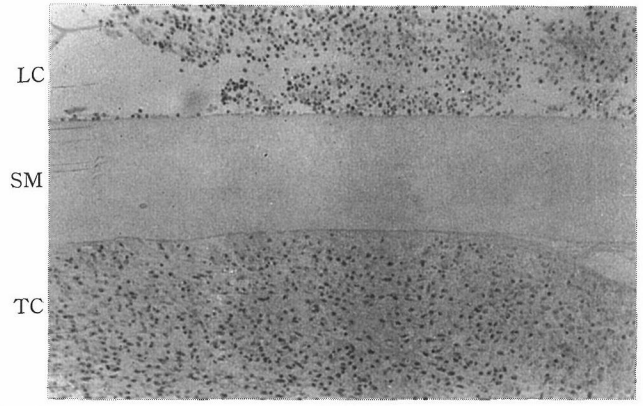

Fig. 17. Photomicrograph of cross section through double diffusion chamber containing the MC tumor plus non-sensitized lymphoid cells. (H.-E. staining)

Histological examination showed that lymphoid cells almost never migrated through the septal membrane, as is shown in Figs. 16 and 17. In the experimental group the target tumor cells were loose by arranged and were undergoing gradual degeneration (Fig. 16), whereas in the control group there was little, if any, degeneration (Fig. 17).

This experiment revealed again that sensitized lymphocytes play a large role in immunological resistance to the growth of MC tumor cells. However, the humoral factor cannot be ignored in the immunological mechanism of tumor transplantation, because the growth of the target tumor was considerably suppressed even in double diffusion chambers with cell-impermeable septal membranes.

DISCUSSION

When host resistance to transplanted tumors is weak, the mechanism of im-

munological resistance may easily be masked by the rapid proliferation of the tumor tissue. The efficiency of the host response can be increased, however, by the use of a very small number of tumor cells for transplantation or by pretreatment with heavily irradiated tumor cells.

The method of immunization reported in this paper used pretreated tumor cells irradiated with 15,000R of ^{60}Co, according to Klein's method. The data presented here demonstrate that MC-induced sarcomas are capable of producing immunity against subsequent inoculation with the same tumor in an inbred strain of mice.

I. Transplantation Immunity against Methylcholanthrene-induced Sarcoma

For many years investigators have been trying to induce transplantation immunity to tumors. In the past such experiments have employed hybrid animals as hosts. However, even when the antigenicity of the tumor is sufficient to produce successful transplantation immunity, the influence of genetic deviations between the hosts and the tumor-bearing animals and of genetic incompatibility due to oft-transplanted tumors must be considered. Recent advances in our knowledge of immunogenetics have shown that it is necessary to study tumors in inbred animals with no genetic differences between tumor and host. Therefore, inbred strains must be used to assure histocompatibility, and tumors induced in these same inbred lines must be employed.

The initial studies were those of Gross[11,12] (1943, '45) and of Foly[8] (1953), who took into account the concept of histocompatibility in the immunology of cancer and demonstrated cancer-specific antigen in mice by the method of cancer transplantation. Later studies which confirmed these findings were reported by Prehn[24,25] (1957, '61), Klein[18,20] (1960, '66), Old[22] (1962), Ushubuchi[34] (1965), Weiss[35] (1964), Alexander[1,2] (1964, '66), Takeda[30,31] (1964, '67) and Kitano[14~17] (1965, '66).

Gross[11] found that the intracutaneous inoculation of small doses of methylcholanthrene-induced sarcomas grown in C3H/He mice regularly induced a state of immunity which prevented the growth of the same tumors when fragments were implanted later. Foley[8] tried to confirm the findings of Gross, using a ligation method.

Prehn[24] demonstrated that dibenzanthracene-induced sarcoma is as capable as MC-induced fibrosarcoma of producing immunity aginst subsequent inoculation of the same tumor in an inbred strain of mice, and that the antigen was peculiar to and specific for its own tumor tissue.

Klein *et al.*[18] produced tumors by intracutaneous injections of methylcholanthrene into the foot pad of mice, then stored the resected tumors to use as antigen. Then, the mice from which the tumors had been removed, together with groups of isologous animals, were repeatedly pretreated with irradiated sarcoma cells and then inoculated with viable tumor cells of the same origin. Untreated isologous mice were also inoculated. An increased resistance to transplanted tumors could be demonstrated in the autochthonous and isologous hosts treated by irradiated tumor cells, but not in the untreated isologous mice. As isologous

mice pretreated with irradiated normal tissue and subsequently inoculated with viable sarcoma cells showed almost no resistance to a given sarcoma, it was considered that this resistance to retransplantation was an immunological phenomenon depending on tumor-specific antigen.

Weiss *et al.*[35,36] (1964) demonstrated resistance to transplanted mammary carcinoma in the original host and showed that the lymph nodes draining the sites of tumor implantation showed lymphoid hyperplasia in the original animals.

Old, Boyse *et al.*[22] (1962) and also Prehn and Main observed that in inbred strains of mice subcutaneous tumors induced by methylcholanthrene or dibenzpyrene have the capacity to immunize the isogenic host against the same tumor. They[22,23] also (1962, '64) summarized the work done on the immunological aspects of experimental tumors.

In the present experiments, the author[17] examined the immunological resistance to tumor transplantation as reflected by the inhibition of growth of MC-induced sarcomas after pretreatment with ^{60}Co irradiated isogenous tumor cells.

The results obtained indicated that mice generally immunized with ^{60}Co irradiated MC sarcoma cells showed a specific resistance to the retransplantation of MC-induced sarcoma. These findings agree with the reports of Klein, Old and Takeda that MC-induced sarcoma contains a specific antigen for its own tissue.

It is important to know whether this immune type of resistance is present equally throughout the whole body or is conspicuous at certain sites.

Recently, Prince *et al.*[26] reported that so-called partial resistance was found in DAB mice with transplantable adenocarcinoma which had originated in the same strain.

The method of transplanting tumor cells into lymph nodes was designed by us for the study of immunity, especially local immunity, to cancer. We found that transplanted tumor appears to grow better in regional lymph nodes draining the sites of immunization than in other areas of the body.

Old *et al.*[23] (1964) showed that BCG infection markedly activated reticuloendothelial function in Swiss mice and greatly inhibited the growth of sarcoma-180 in the host. Our experiment showed that in mice infected with BCG resistance to MC-induced sarcoma was enhanced.

II. Diffusion Chamber Method in Research on Transplantation Immunity of Tumors

Since Algire[3] first (1954) designed the diffusion chamber as a means of studying cellular antibodies against transplanted tumor, it has been employed in analyzing the immunological response.

Algire *et al.*[4,5] found that mammary tumors of C3HBA mice and Harderian glands of the same strain of mice placed in diffusion chambers with HA filters grew adequately when the chambers were inserted into the peritoneal cavities of mice immunized with the same tissue. However, they were destroyed immediately when the chambers were inserted into the subcutaneous tissue of the immunized mice. Futhermore, they found that spleen cells from mice immunized by the same cells placed in cell-impermeable chambers were destroyed within

a week. Their diffusion chamber study is an original method of analyzing the action of cellular and humoral antibodies against transplanted tissue.

Later, Amos *et al.*[6] (1959) implanted cell-impermeable diffusion chambers containing DBA/2 lymphoma cells into the peritoneal cavities of mice and observed that, after a slight initial fall, the number of ascites tumor cells increased rapidly in the chamber. In 75% of the mice DBA/2 lymphoma in the chambers degenerated rapidly when anti-DBA/2 lymphoma serum was injected repeatedly into the peritoneal cavity. On the basis of these facts, they concluded that humoral antibodies could diffuse into the chamber through the cell-impermeable filter.

Gabourel[10] (1961) found that the growth of L-fibroblasts in diffusion chambers implanted in the peritoneal cavity of C3H mice was inhibited by sensitizing the host with L-fibroblasts. He also found that the serum from C3H mice sensitized with L-fibroblasts inhibited the growth of the same cells in diffusion chambers. He concluded that the humoral factor, presumably antibodies, may play an important role in the rejection of transplants of the same kind of cell.

With the unique chamber method used in the present study the author demonstrated that in mice immunized with MC tumor cells irradiated by ^{60}Co, the growth of the same tumor in the diffusion chamber was inhibited regardless of the size of the pores of the filter. He also observed that a large number of lymphocytes are attracted to the tumor cells in diffusion chambers inserted into immunized mice.

On the basis of the results of their experiments with diffusion chambers, Algire *et al.*[5] (1955) concluded that cellular antibodies are carried by lymphocytes.

However, Amos[6], Gabourel[10] and the present author[15] have shown that tumor cell growth is inhibited in both cell-permeable and cell-imperneable diffusion chambers inserted into immunized mice, and therefore that immunological inhibition of tumor growth does not require immunized lymphocytes to be in direct contact with the target tumor cells.

III. Cytotoxic Factors of Immunized Lymphocytes

Recently it has been shown experimentally that lymphocytes from immunized animals are attracted to and cause the cytolysis of target cells *in vitro*. The question arises as to whether or not contact between the immunized lymphocytes and the target cells is essential for the cytolysis of the target cells to occur.

Rosenau *et al.*[28,29] (1961, '62) studied the effects of sensitized lymphocytes of BALB/c mice on homologous cells of the L-strain obtained from C3H mice. They found that sensitized lymphocytes clustered around the L-cells, and L-cells consequently showed cytolysis. Their observation emphasizes that cellular factors play an important role in immune processes.

Later, Koprowski *et al.*[21] (1962) confirmed Rosenau's observation by employing lymphocytes from lymph nodes of inbred rats sensitized with guinea pig cord tissue. They also observed that sensitized lymphocytes clustered around the target cell, and called this phenomenon "contactual agglutination" of the lympho-

cyte. The contactual agglutination test seems to provide an opportunity for investigation of cellular response in tissue culture systems.

Taylor *et al.*[32] (1963) also confirmed that both homologous and heterologous spleen cells, sensitized against L-strain fibroblasts had the same effect on L-strain fibroblasts *in vitro*, and then demonstrated that sensitized lymphocytes must be alive to cause the immunological response. Analogous results were obtained by Hanaoka[13] (1962) and Tanaka[33] (1966).

These results of *in vitro* cell culture studies prove that sensitized lymphocytes surround the target cells and contact them to cause cytolysis. However, the question remains as to whether contact with sensitized lymphocytes in indispensable for the cytolysis of the target cells.

Wissler *et al.*[37] (1957) mentioned that sensitized lymphocytes may release a cytotoxic substance in the neighborhood of tumor cells to destroy them.

Algire[3,5] (1954, '55) suggested that the diffusible substance released by immunized lymphocytes plays an important role in the mechanism of transplantation immunity. Amos[6,7] (1959, '60) thought that the cytotoxic effect of sensitized lymphocytes might due to some enzyme.

The experiments with cell-permeable single diffusion chambers and double diffusion chambers with cell-permeable filters as septal membrane suggest that target cells are destroyed by contact with sensitized lymphocytes. However, the experiments with cell-impermeable single diffusion chambers and double diffusion chambers with cell-impermeable filters as septal membrane indicate that direct contact with sensitized lymphocytes is not indispensable for the injury of the target cells, and that some diffusible substance or humoral antibodies released by the lymphocytes can cause cytolysis of the target cells. Whether or not the diffusible substance (Algire), cytotoxic substance (Wissler *et al.*) are identical is a problem to be solved in the future.

SUMMARY

The possible influence of the immunological mechanism in transplantable tumors was investigated by examining tumor inhibiting factors in mice sensitized systemically or locally by methylcholanthrene-induced sarcoma. A unique diffusion chamber technique was used to determine whether transplanted MC-induced sarcomas in mice immunized by the same tumor are destroyed by cellular antibodies, by humoral antibodies, or by both.

1. Inbred DDD-s mice were injected with tumor cells irradiated with ^{60}Co (15,000R). They showed a marked immune resistance to subsequent transplantation of the same tumor in the subcutaneous tissue, whereas no resistance was observed in animals similarly pretreated with normal mouse tissue.

2. The author designed a method of transplanting tumor cells into the inguinal lymph nodes of mice for the purpose of testing the local immunity in the regional lymph node draining the site earlier injected with tumor cells.

3. Immunological resistance to the growth of the transplanted tumor proved to be stronger in this regional lymph node than in other lymph nodes.

4. The author designed the diffusion chamber method of the Chest Disease Research Institute, National Kyoto University, and both single and double diffusion chambers were used to determine whether transplanted tumor is destroyed by cellular or by humoral antibodies.

5. The single diffusion chamber method im mice sensitized by the same tumor showed a remarkable immune resistance to target tumors in both cell-permeable and cell-impermeable diffusion chambers, whereas no resistance was observed in mice sensitized by normal tissue. Experiments with cell-permeable chambers suggested that tumor cells are destroyed by direct contact with sensitized lymphocytes. However, experiments with cell-impermeable chambers showed that contact with sensitized lymphocytes is not indispensable for injury of the tumor cells.

6. The double diffusion chamber method with cell-permeable MF used as the septal membrane showed again that injury of the target tumor seemed to be due to contact with sensitized lymphocytes, but when cell-impermeable MF was used as the septal membrane target cell injury occured even without direct contact with sensitized lymphocytes.

7. Therefore, it can be concluded that when lymphocytes can pass through the membrane of the diffusion chamber, target tumor cells are destroyed by contact with sensitized lymphocytes, but when they cannot pass through the membrane, sensitized lymphocytes plays a large role in the immune response.

ACKNOWLEDGMENTS

The author wishes to express his sincere thanks to Professor T. Nagaishi and Dr. Y. Okada for their continual guidance and valuable discussion on this problem. Thanks are also due to Professor S. Shimizu and Mr. R. Katano for extending the facilities for γ-ray irradiation to his work.

REFERENCES

(1) P. Alexander and E. J. Delorme, *Lancet*, 18, 117 (1964).
(2) P. Alexander, Z. B. Mikulska and C. Smith, *J. Nat. Cancer Inst.*, 36, 29 (1966).
(3) G. H. Algire, R. T. Prehn and J. M. Weaver, *J. Nat. Cancer Inst.*, 15, 509 (1954).
(4) G. H. Algire, R. T. Prehn and J. M. Weaver, *J. Nat. Cancer Inst.*, 15, 493 (1954).
(5) G. H. Algire, R. T. Prehn and J. M. Weaver, *J. Nat. Cancer Inst.*, 15, 1737 (1955).
(6) D. B. Amos and J. D. Wakefield, *J. Nat. Cancer Inst.*, 22, 1077 (1959).
(7) D. B. Amos, *Ann. N. Y. Acad. Sci.*, 87, 273 (1960).
(8) E. J. Foley, *Cancer Res.*, 13, 835 (1953).
(9) J. D. Gabourel and K. E. Fox, *Cancer Res.*, 19, 1210 (1959).
(10) J. D. Gabourel, *Cancer Res.*, 21, 506 (1961).
(11) L. Gross, *Cancer Res.*, 3, 326 (1943).
(12) L. Gross, *J. Immunol.*, 50, 91 (1945).
(13) M. Hanaoka and K. Notake, *Ann. Rep. Ins. Virus Res. Kyoto Univ.*, 5, 134 (1962).
(14) M. Kitano, S. Ikeda, M. Ito, F. Todoroki, Y. Orita and Y. Okada, *Proc. Jap. Cancer Assoc.*, 24, 73 (1965).
(15) M. Kitano, S. Ikeda, M. Ito, F. Todoroki and Y. Okada, *Proc. Jap. Cancer Assoc.*, 25, 130 (1966).
(16) M. Kitano, S. Ikeda, M. Ito, F. Todoroki and Y. Okada, *J. Jap. Soc. Cancer Ther.*,

1, 44 (1966).
(17) M. Kitano and Y. Okada, *J. Jap. Soc. Cancer Ther.*, **2**, 51 (1967).
(18) G. Klein, H. O. Sjögren, E. Klein and K. E. Hellström, *Cancer Res.*, **20**, 1561 (1960).
(19) G. Klein and H. O. Sjögren, *Cancer Res.*, **20**, 452 (1960).
(20) G. Klein, *Internat. Cancer Congr.*, **9**, 289 (1966).
(21) H. Koprowski and M. V. Fernandes, *J. Exp. Med.*, **116**, 467 (1962).
(22) L. J. Old, E. A. Boyse, D. A. Clarke and E. A. Carswell, *Ann. N. Y. Acad. Sci.*, **101**, 80 (1962).
(23) L. E. Old and E. A. Boyse, *Ann. Rev. Med.*, **15**, 167 (1964).
(24) R. T. Prehn and J. M. Main, *J. Nat. Cancer Inst.*, **18**, 769 (1957).
(25) R. T. Prehn, *Ann. N. Y. Acad. Sci.*, **94**, 107 (1961).
(26) J. E. Prince, J. C. Fardon, L. G. Nutini and G. S. Sperti, *Cancer Res.*, **17**, 312 (1957).
(27) L. Révész, *Cancer Res.*, **20**, 443 (1960).
(28) W. Rosenau and H. D. Moon, *J. Nat. Cancer Inst.*, **27**, 471 (1961).
(29) W. Rosenau and H. D. Moon, *Lab. Invest.*, **11**, 1260 (1962).
(30) K. Takeda and M. Aizawa, *SAISHIN-IGAKU*, (Japan) **19**, 474 (1964).
(31) K. Takeda, *Gen. Ass. Jap. Med. Congr.*, **17**, 22 (1967).
(32) H. E. Taylor and C. F. A. Culling, *Lab. Invest.*, **12**, 884 (1963).
(33) S. Tanaka, K. Orita, K. Sato and N. Sato, *Igaku no Ayumi*, **56**, 590 (1966).
(34) I. Ushubuchi, *J. Cancer Immunopathology*, (*Japan*) **1**, 71 (1965).
(35) D. E. Weiss, L. T. Faulkin and K. B. De Ome, *Cancer Res.*, **24**, 732 (1964).
(36) D. E. Weiss, M. A. Attia and K. B. De Ome, *Cancer Res.*, **25**, 451 (1965).
(37) R. W. Wissler and M. H. Flax, *Ann. N. Y. Acad. Sci.*, **69**, 773 (1957).