CLINICAL APPLICATION OF DIFFUSION CHAMBER TECHNIQUE IN THE FIELD OF CANCER TREATMENT

by

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Received for Publication, Jul. 20, 1963

INTRODUCTION

It is well known that H. A. type millipore filter permits the passage of polymolecular substances such as albumin and globulin but not of cells. Using a millipore filter, ALGIRE et al. devised a diffusion chamber method which made it possible to cultivate cells in a chamber inserted into a host body without mingling the cells with host cells. In this study, by use of the diffusion chamber, the following points were investigated :

i) Behaviors of various cells in the diffusion chambers inserted into auto-, iso- or homologous hosts.

ii) Growth of cells in the diffusion chambers inserted into heterologous hosts conditioned by X-ray irradiation and/or predonisolone administration.

iii) Possible interaction between human cancer cells and homogenate of the regional lymph nodes in the diffusion chamber inserted into an autologous host.

iv) Availability of the diffusion chamber method as a sensitivity test for anticancer agents.

MATERIALS and METHODS

i) *Diffusion chamber*

An acrilite ring (meta-acril acid methyl ester polymer manifactured by the MITSUBISHI RAYON Co. Ltd.), measuring 14 or 16mm in outside diameter, 10 or 12mm in inside diameter, 2 or 3mm thick, was used as a skelton of the chamber. An access hole large enough to permit passage of a hypodermic needle was drilled into the wall of the ring. The top and bottom of this ring were closed by discs of H. A. type millipore filter, manifactured by the Millipore Corporation, Watertown, Mass., U. S. A., and sealed with a cement, 5 percent solution of acrilite in acetic acid. After having secured complete adhesion of the discs and the ring, the completed diffusion chamber was washed by running water for a period of 12～24 hours in order to expel obnoxious materials, especially acetic acid.

ii) *Sterilization of the chamber*

The following methods were used :

a) autoclave-sterilization (10～15 minutes)

b) formaline gas sterilization (over 24 hours)

c) 70% alcohol immersion (10～15 minutes)

iii) *Culture medium*

Physiological saline solution, RINGER's solution and supernatant of donor's ascites or

human serum were used to make culture media. The main culture media were ;

a) mixture of supernatant of donor's ascites or human serum and the same volume of GEY'S solution.

b) mixture of 3 portions of physiological saline or RINGER'S solution, 1 portion of supernatant of donor's ascites or human serum and 1 portion of (1 : 1) chickembryo extract[25].

iv) *Chamber procedure*

After the sterile chamber had been filled with the cell suspension or a solution through the access hole, the hole was sealed with 20～30% filter acetone solution. When a tumor fragment larger than the access hole was to be implanted, the ring was sealed with a disc at the bottom only. After having implanted the tumor fragment into the chamber, the top was sealed. Then the chamber was filled with the medium introduced through the access hole, which was sealed in the same way. When cell numbers were examined, a known volume of medium containing counted ascites tumor cells, or counted fine cell suspension of solid tumor, was introduced into the sterile chamber through the access hole. The cell number was regulated, by diluting the cell suspension with an appropriate medium, so that each chamber contained about 2 or 3 millions cells.

In other cases, coarse cell suspension was simply introduced by the same procedures.

Recipient animals, C 3H and dd strain mice or Wistar strain and other hybrid rats, were anesthetized by intraperitoneal injection of sodium pentobarbital, the dose of which was approximately 0.02 cc for a mouse (ca. 20gm in body weight) or 0.2 cc for a rat (100～150gm in body weight). After the skin surface was sterilized with 5% tinctura jod. diluta, the abdominal cavity was opened, the chamber containing cells to be tested was inserted and then the peritoneum and the skin was closed with a continuous silk suture.

v) *Cell suspension*

Coarse cell suspension was prepared by squeezing a solid tumor tissue through a 20 mesh cytosieve out into a culture medium containing a small amount of Tween 80, one of the surface active agents. In order to prepare fine cell suspension[49] out of the coarse cell suspension, a modified bubbling method was employed. The construction of the ap-

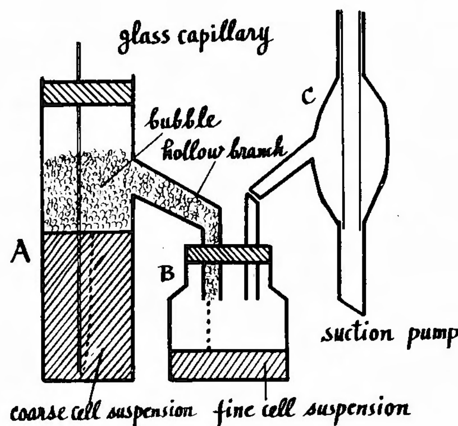


Fig. 1 Diagram of apparatus of modified bubbling method

paratus for this method is illustrated in Figure 1. The single cells in the coarse cell suspension in glass tube A were carried through the hollow branch into bottle B on the air bubbles induced by negative pressure produced by suction pump C.

vi) *Tumors*

Ascites tumors used in this study were MH 134 ascites hepatoma, EHRLICH ascites carcinoma, Sarcoma 180 and YOSHIDA sarcoma.

Solid tumors used were MC/4, 5 carcinoma (MIYAWAKI)[29], NF sarcoma, BASHFORD carcinoma 63 and gastric cancers and other human cancers.

vii) *Animals*

C3H strain mice were used for transplantation of MH 134 ascites hepatoma; dd strain mice for EHRLICH ascites carcinoma, Sarcoma 180, MC/4, 5 carcinoma, NF sarcoma and BASHFORD carcinoma 63; Wistar strain and other hybrid rats were used for YOSHIDA sarcoma; volunteer patients were used for human cancers. C3H and dd strain mice and Wistar strain rat were supplied from the animal center of Kyoto University.

viii) *Calculation of cell numbers*

3 ml. of 0.1 M citric acid containing 0.05% crystal violet was pipetted into a 25 ml. beaker. The surface of the chamber removed from abdominal cavity was at first washed well in running water. Being immersed into the solution of the beaker, the chamber was then opened by cutting around a filter membrane with the point of a pipette. The beaker was shaken for 30 minutes at 37°C or for about an hour at room temperature, until the cells were broken and the nuclei were separated from them into a homogeneous suspension. Numbers of nuclei of tumor cells were counted in a hematocytometer.

ix) *Homogenate of lymph nodes*

Regional and remote lymph nodes were removed from MH 134 bearing mice. The lymph tissue was suspended in three volumes of supernatant of the donor's ascites. Lymph nodes from cancer patients were suspended in the autologous serum in the same way. The suspendings were homogenized in POTTER's homogenizer by rotating the cyinder twenty or thirty times by hand.

x) *Anticancer agents*

Anticancer agents used in sensitivity tests were as follows; Thio-TEPA (Tespamin), Cytoxan (Endoxan), Mitomycin C and Hg-Hematoporphyrin-Na (Merphyrin). Immediately before introducing the cell suspensions into the chamber, these agents were added to cell suspension. Final concentrations of these agents in all suspensions were adjusted correspondingly to usual clinical concentrations of the agents in blood that is, Tespamin: $1/5\gamma$/cc, Endoxan: 4γ/cc, Mitomycin C: $2/25\gamma$/cc, Merphyrin: 1γ/cc.

xi) *Sampling and examination of chamber contents*

The surface of the chamber taken out from the peritoneal cavity was washed carefully in running water in order to remove fibrous membrane sticking on the outside of the chamber. The chamber was opened by removing one of the filter membranes and the content of the chamber was aspirated and made into smear preparations. When the content of the chamber was found to be partially cloted, the clot was minced with the point of a needle before aspiration. GIEMSA's staining was usually employed. However, some specimens were stained with Hematoxylin-Eosin solution. A histological examination was then

performed.

RESULTS

*** Host reaction**

A few days after the chamber filled with physiological saline solution had been inserted into the peritoneal cavity, thin translucent fibrous membrane was observed covering the outside of the chamber. Thereafter, the membrane gradually became thicker, but not thicker than 1/10～1/20mm. In the fibrous membrane, vascularisation occurred within 30 days after insertion of the chamber (Photo. 1). Slight inflammatory reaction was usually observed around the chambers. When the cells, especially of heterografts, in the chamber underwent degeneration or were contaminated by bacteria, heavy local inflammation took place around the chamber with a continuous growth of the fibrous membrane. Less than 10% of the experimental animals succumbed to operative procedures of inserting the chambers. In cold circumstances animals were less resistant to operative procedures. Once having tolerated the operative procedures, animals survived for a long period of time, bearing the chambers in their peritoneal cavity.

*** Passage of peritoneal fluid into the chambers**

After the chambers filled with physiological saline solution had been inserted into the peritoneal cavity of EHRLICH ascites carcinoma bearing mice, the content of the chambers were examined by paper electrophoresis and the entry of fractions of protein into the chambers was observed as illustrated in Figure 2 which indicated that polymolecular protein freely passed through the filter membrane. Densitometry revealed that, 24 hours after inserting the chamber, albumin content was a little higher than that of supernatant of ascites, while globulin stayed at the same level.

Fig. 2 Entry of proteins into diffusion chambers inserted into peritoneal cavity of EHRLICH ascites carcinoma bearing mice
(veronal buffer, PH. 8.6, $\mu=0.05$, 200 volts, 8hours)

*** Leakage of tumor cells from the chamber**

ISHIBASHI[21] and HOSOKAWA[19] reported that a very single cell of YOSHIDA sarcoma could be transplanted. If adhesion of the filter to the ring or sealing of the access hole was imperfect, the tumor cells in the chamber could escape from the gaps. On such an occasion the results of experiments should be discarded. Therefore, in order to test if leakage of tumor cells would occur or not, YOSHIDA sarcoma were transplanted in chambers in hybrid rats. Provided that some cells leaked from the chambers which had some struc-

tural imperfection, the hybrid rats in which the chambers were inserted should have developed a tumor. It was revealed that no rats developed the tumor 1, 2 or 3 months after inserting the chambers as indicated in Table 1.

Table 1 Growth of YOSHIDA sarcoma in hybrid rats in which YOSHIDA sarcoma were transplanted in chambers

No. \ month	1 M	2 M	3 M
1	—	—	—
2	—	—	—
3	—	—	—
4	—	—	—
5	—	—	—

* Entrance of host cells into the chamber

(Observation of degenerative change of EHRLICH ascites carcinoma cells)

EHRLICH ascites tumor cells grew slowly in the chambers in dd mice. The cause of the slow growth was perhaps due to low viability of the cells. Tumor cells with feeble viability would be damaged more markedly by a homograft reaction than those with strong viability. Tumor cells with low viability, which come into contact with host cells, may be degenerated easily by homograft reaction. Therefore, suppose the host cells migrated into the chamber containing EHRLICH ascites carcinoma cells, the tumor cells would be expected to be more degenerative than those in a perfectly closed chamber. In the present experiments more degenerative changes of tumor cells were not detected histologically in any chambers.

* Growth curve of a few experimental tumors

Eight experimental tumors and various human cancers were examined. Growth curves of MC/4, 5 (MIYAWAKI[29]) and EHRLICH ascites carcinoma were illustrated in Figure 3.

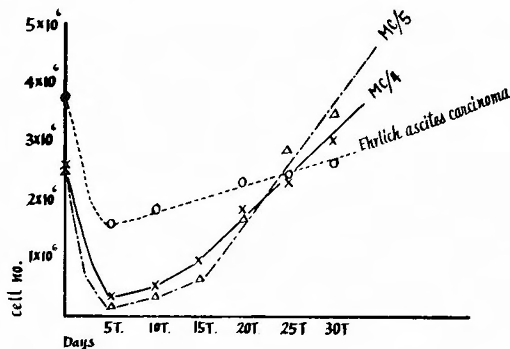


Fig. 3 Growth curve of homografts in dd mice

MC/4, 5 grew rapidly after the initial drop, whereas EHRLICH ascites carcinoma grew slowly in dd mice. AMOS and WAKEFIELD[5] reported the initial drop was hardly affected by either numbers of cells, the adhesives used for sealing, the sterilization procedures or the materials of the chamber. In the present experiments, conditioning of host animals by either X-ray irradiation or predonisolone administration gave little effect to the initial drop.

And the initial drop was also not affected by culture media. When the chambers containing EHRLICH ascites tumor cells were inserted into the same tumor bearing mice, beginning of the initial drop was retarded and its slope became flat. As far as these three tumors were concerned, an attempt to calculate generation times of the tumor cells was not successful because the logarytmic phase of these tumor growths in chambers was not so clear. By well designed chambers and adoption of appropriate genotype hosts, generation times of many tumors could be calculated as AMOS and WAKEFIELD reported.

*** Chronological changes of tumors**

Auto-, Iso-, and homologous transplants were capable of surviving or growing in chambers for a long period of time. The fact that homografts survive or grow in chambers may be attributed generally to both autonomy and viability of tumor cells themselves. However, there were some differences of cellular behavior between various transplanted tumors in chambers. This might be caused by difference of genotypes of these tumors.

i) *MH 134 ascites hepatoma*

SATO et al. had induced MH 134 ascites hepatoma in C3H mouse in 1953. The tumor used in this experiment was obtained from TAKEDA Chemical Industries Ltd. In the chamber method this tumor was transplanted in C3H mice as isografts and also showed the initial drop. After the initial drop, the tumor grew progressively in chambers, keeping the same cytological findings as seen at the time of the transplantation (Photos. 2, 3).

ii) *EHRLICH ascites carcinoma*

LOEWENTHAL and JAHN established the tumor from a spontaneous mammary cancer in a hybrid mouse in 1930. This tumor grew very slowly in chambers. Such cytological findings as the presence of more degenerative cells and smaller cells in size than that at the time of transplantation might be in accord with the slow rate of the tumor growth (Photos. 4, 5).

iii) *MC/5*

MIYAWAKI, a member of our laboratory, established the tumor from the spontaneous mammary adenocarcinoma in dd strain mouse in 1959. The histological picture of the tumor at the time of removing from the donor was indicated in Photo. 6. In making coarse cell suspension many tumor cells were damaged due to mechanical manipulation of crushing the tumor mass (Photo. 7). They soon became detached from viable tumor clumps and then became components of clots in the chamber. Rapid proliferation of the cells successively took place from the remaining viable cells (Photos. 8, 9).

iv) *NF sarcoma*

NAKAHARA and FUKUOKA established the tumor from a hybrid mouse in 1948. The tumor used in this experiments was obtained from the SHIONOGI and Co. In some experiments of this tumor, sticks of the tumor as well as coarse cell suspension were used. In both cases the tumor cells grew as well as other homografts. The cytological findings of the tissue preparations 15 days after sealing into chambers indicated no signs of degeneration, compared with that at the time of transplantation (Photos. 10, 11). The tumor cells from the chambers were, however, generally larger than that at the beginning of the experiment, and indicated the picture of proliferation.

v) BASHFORD *carcinoma 63*

BASHFORD established the tumor from a mouse in 1912. The tumor used in the experiment was obtained from the SHIONOGI and Co. Growth of the tumor in chambers was as that of EHRLICH ascites carcinoma in dd recipient mice. The cytological findings 10 days after transplantation indicated more degenerative changes than that at the beginning of the experiment. However, normally shaped tumor cells among the degenerative ones indicated slow porliferation (Photos. 12, 13).

vi) *Sarcoma 180 (ascites form)*

WOOD and PFIME discovered the neoplasm in a hybrid mouse as mammary adenocarcinoma, but after many successive subcutaneous transplantations, it became a sarcoma and does not change. The cytological findings of the tumor cells 20 days after transplantation in chambers indicated that there was a few slightly deformed cells, compared with that at the beginning of the experiment (Photos. 14, 15).

vii) YOSHIDA *sarcoma*

YOSHIDA extablished the tumor artificially from a hybrid rat in 1943. The tumor used in this experiment was obtained from the TAKEDA Chemical Industries Ltd. The cytological findings of the tumor cells 15 days after transplantation in chambers indicated that a few of the tumor cells became large in size and elliptical in shape, compared with that at the beginning of the experiment (Photos. 16, 17).

viii) *Human cancer*

Many investigators[22)32)] reported on autotransplantation of human cancers and they consistently emphasized spontaneous regression of transplanted tumor mass. Although some parts of cells of autografts in diffusion chambers, so far as observed within a month, survived, larger parts of cells disappeared away within a week (Photos. 18, 19). It is very curious that autografts finally regressed gradually, even though at the earlier period of transplantation they grew to some extent, in spite of progressive growth of original tumor in situ. The sticks of a metastatic seminoma which had been sealed into chambers indicated the degenerative changes 21 days after transplantation (Photos. 20, 21).

*** Heterotransplantation by conditioning of hosts**

ALGIRE et al.[3)] reported that heterografts in chambers had survived a little longer than that of subcutaneous or intraperitoneal transplantation. Since MURPHY had succeeded in heterologous transplantation of EHRLICH ascites carcinoma into X-ray irradiated rats, by conditioning hosts in the same way many investigators have reported on successful heterologous transplantation. TOOLAN[46)] investigated various methods of conditioning. To get prolongation of survival time or successive growth of heterografts in chambers conditioning of hosts by either or both of predonisolone injection and X-ray irradiation was performed as follows ;

1) dd strain mice of about 20gm body weight in which YOSHIDA sarcoma was going to be transplanted were conditioned by the following methods ;

a) Subcutaneous predonisolone administration in dose of 2mg/kg, 4mg/kg or 8mg/kg daily during 14 days before and after transplantation.

b) X-ray irradiation in dose of 200r, 250r or 300r 1 or 3 days before transplantion. (160 KVP, 3 ma, 0.4mm Cu-0.5mm Al filters, TSD 40cm, 11.4/min).

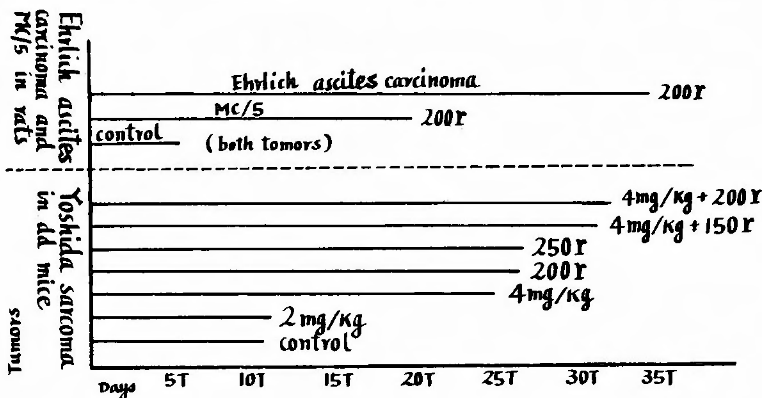
c) Combination of both predonisolone administration in dose of 2mg/kg or 4mg/

kg and X-ray irradiation in dose of 150r or 200r in the same manner as a) and b).

2) Wistar strain rats of about 100mg body weight, in which EHRLICH ascites carcinoma and MC/5 were going to be transplanted were conditioned by X-ray irradiation in doses of 200r 1 or 3 days before transplantation was used.

In these experiments five animals were used as a group. In all cases, the cytological findings of the transplanted tumor cells, especially of the nuclei, were taken as criteria for the effect of conditioning. Marked prolongation of survival time was noticed shown in Table 2. But no successive development of cell division was found. It was not practi-

Table 2 Life prolongation of heterografts in diffusion chambers by predonisolone injection, X-ray irradiation and their combination to host animals



cal to condition the host animals in doses of 8mg/kg of predonisolone or 300r irradiation, because in these doses in spite of high mortality rate of animals, the effects of conditioning were not neccessarily better than those in the other doses, referring to the cytological findings of tumor cells transplanted to the remnant animals.

* Influences of homogenate of regional lymph nodes to the original tumor cells

Many investigators have approved of radical dissection of regional lymph nodes in radical surgery of human cancers, but it might be open to discussion yet. In order to clarify the significance of regional lymph nodes as a barrier and/or their oncolytic action to cancer cells, the following experiments were performed. One volume of the homogenate of lymph nodes was added to three volumes of the coarse cell suspension of the tumors. The tumor cells mixed with the homogenate in chambers were inserted intraperitoneally as isografts or autografts.

i) *Animal experiment*

MH 134 ascites hepatoma, one of the most metastasizable tumors, is used frequently in the study of metastasis, because of its metastatic mechanism is said to be close to that of human cancers. Retroperitoneal lymph nodes of MH 134 ascites hepatoma bearing C3H mouse were resected and then hemogenated. Both 7 days and 14 days after transplantation, isografts mixed with the homogenate were taken out from the chambers and examined. So far as the results obtained, the homogenate of the lymph nodes seemed not to have any remarkable influence on the growth of the tumor cells (Table 3). As a control in this

Table 3 Influences of lymph node homogenate to the original tumor cells (MH 134 ascites hepatoma)

lym. \ no. \ days	no.	7 T	14 T	result
regional (retroperitoneal) lymph node	1	+	+	negative
	2	+	+	negative
	3	+	+	negative
remote (inguinal) lymph node	1	+	+	negative
	2	+	+	negative
	3	+	+	negative

+ : viable tumor cells detectable

experiment homogenate of inguinal lymph nodes resected from 3 or 4 C3H mice was used.

ii) *Clinical application*

By the same method in the animal experiments, behavior of original gastric cancer cells mixed with the homogenate of regional lymph nodes was observed 7 days after autotransplantation (Table 4). As a control the inguinal or mesenterial lymph nodes which grossly appeared normal were used (Control II in Table 3). As another control the tumor cells were used alone (Control I in Table 3). Any finding suggesting histoimmunological significance of the lymph nodes was also not found as in the animal experiments.

Table 4 Influences of lymph node homogenate to the original tumor cells (human cancer)

name	age	sex	histologinal findings	control I	regional lymph node	control II remote lymph node	result
K. K.	32	f	adeno-ca.	±	—	—	positive ?
N. S.	53	m	undiff. ca.	+	+	+	negative
O. T.	54	f	adeno-ca.	+	+	+	negative

— : difficult to detect tumor cells
± : degenerative tumor cells detectable
+ : viable tumor cells detectable

* Sensitivity test using a few representive anticancer agents

As well known, each tumor dces not necessarily respond to various anticancer agents in the same way. Therefore, a sensitivity test is one of the important problems in studies of anticancer agents. In chamber method, it may be considered that anticancer agents react to the tumor cells in combination with peritoneal fluid which passes freely through the filter membranes. Keeping this in mind, attempts were made to test sensitivity of tumors to anticancer agents by this method. The anticancer agents tested were Mitomycin C, Tespamin, Endoxan and Merphyrin, and then respective doses were 2/25γ/cc, 1/5γ/cc, 4γ/cc and 1 γ/cc. A diluted solution of an agent of a known concentration was mixed with the cell suspension at the beginning of the experiment.

(*A*) *Sensitivity test of experimental tumors*

These experiments were performed preliminarily before the clinical application. Considering variety of the results presumably due to differences of natural resistance in indi-

vidual host animals, the average of the data obtained from some separate experiments was taken. In one experiment, about 20 animals were used, because prolonged manipulation time by using simultaneously more than 20 animals prompts degenerative changes of collected tumor cells. The results of the effect of agents were evaluated 7 or 10 days after transplantation.

i) *MH 134 ascites hepatoma*

Mitomycin C was slightly effective as indicated in Table 5. The result of this ex- periment was similar to the result of UCHIDA, a member or our laboratory.

ii) EHRLICH *ascites carcinoma*

Mitomycin C and Merphyrin were both effective as indicated in Table 6. SUGIURA[43)44)] reported that the tumor was destroyed completely by Mitomycin C and inhibited markedly by Thio-TEPA, but many investigators of our laboratory noticed that EHRLICH ascites carcinoma (our strain) was slightly effected by Thio-TEPA. Merphyrin was effective, contrary to the result reported by MIZUNO et al[31)].

iii) *MC/5*

Mitomycin C was effective as indicated in Table 7. The result was similar to the results obtained from the therapeutic experiments performed by the investigators of our laboratory.

iv) *NF sarcoma*

Mitomycin C and Merphyrin were both effective as indicated in Table 8. FUKUOKA[15)] indicated that Mitomycin C was effective in 75.2%, Thio-TEPA 84.2 % and Merphyrin 0 % of tumor bearing mice. Sensitivity of this tumor to Tespamin and Merphyrin was not consistent with the result mentioned above, moreover Merphyrin was proved to be an effective agent.

v) BASHIFORD *carcinoma 63*

Table 5

Sensitivity test of MH 134 ascites hepatoma to several anticancer agents

agents \ days	2 T	7 T	12T	20T	result
control	++	++	++	++	—
Mitomycin C	+	±	+	++	effctive
Tespamin	+	++	++	++	ineffective

— : difficult to detect tumor cells
± : degenerative tumor cells detectable
+ : viable tumor cells detectable
++ : many viable tumor cells detectable

Table 6

Sensitivity test of EHRLICH ascites carcinoma to several anticancer agents

agents \ days	5 T	10T	15T	25T	result
Control	+	+	+	+	—
Mitomycin C	+	—	+	+	effective
Tespamin	+	±	+	+	ineffective ?
Merphyrin	+	—	+	+	effective

The meaning of —, ±, + and ++ are same as shown in Table 5

Table 7

Sensitivity test of MC/5 to several anticancer agents

agents \ days	5 T	10T	15T	20T	result
Control	±	+	+	++	—
Mitomycin C	±	—	+	++	effective
Tespamin	±	±	+	++	ineffective ?

The meaning of —, ±, + and ++ are same as shown in Table 5

Table 8

Sensitivity test of NF sarcoma to several anticancer agents

agents \ days	5 T	10T	15T	20T	result
Control	±	±	+	+	—
Mitomycin C	±	—	—	+	effective
Tespamin	±	±	+	+	ineffective
Nerphyrin	±	—	—	+	effective

The meaning of —, ±, + and ++ are same as shown in Table 5

Mitomycin C and Tespamin were both rather stimulous or promotive to tumor growth in the early period of the experiment and not effective in any stadium as indicated in Table 9. SUGIURA[43)44)] reported that the tumor was inhibited moderately by both Mitomycin C and Tespamin.

vi) *Sarcoma 180 (ascites form)*

Mitomycin C and Tespamin were both effective as indicated in Table 10. SUGIURA [43)44)] reported the tumor was destroyed completely by Mitomycin C and inhibited moderately by Tespamin.

vii) YOSHIDA *sarcoma*

Mitomycin C and TESPAMIN were both effective as indicated in Table 11. USUBUCHI[47)] reported the tumor was effected by Mitomycin C when given more than 500mg/kg. ISHIDATE[23)] reported life prolongation was observed when the tumor bearing rats were treated with Tespamin.

(*B*) *Sensitivity test of human cancer*

As the results of sensitivity tests of various experimental tumors were generally parallel with that of therapeutic experiments of many investigators, clinical application of this sensitivity test was attempted. Various agents were taken for human cancers, especially for gastric cancers. In several cases, cytological findings were studied 7 days, 14 days and 21 days after transplantation. However, it became clear that sensitivity tests can only be determined by cytological examination 7 days after transplantation. Effective agents obtained by our ordinary method of chamber technique are indicated in Table 12. In cases of gastric cancers, Mitomycin C was effective in 22% and alkylating agents in 40% of the cases. GOMIBUCHI[17)] reported Mitomycin C was effective in 45.5% and Tespamin in 53.6% of human cancers by use of I. N. K. method[52)]. In each tests alkylating agents were more effective than Mitomycin C. It is worthy to note that there were no common effective therapeutics among the tumors which originated from the same organ and indicated similar histological findings. In one case, stimulous effect of therapeutics was noted temporarily, but in the long process of time, this phenomenon disappeared and then tumor

Table 9

Sensitivity test of BASHFORD carcinoma 63 to several anticancer agents

agents \ days	7 T	10T	15T	20T	result
Contol	±	+	++	++	—
Mitomycin C	++	++	++	++	stimulous?
Tespamin	++	++	++	++	stimulous?
Merphyrin	±	+	++	++	ineffective

The meaning of —, ±, + and ++ are same as shown in Table 5

Table 10

Sensitivity test of Sarcoma 180 to several anticancer agents

agents \ days	5 T	10T	15T	20T	result
Control	+	+	+	+	—
Mitomycin C	±	±	±	—	effective
Tespamin	+	±	±	—	effective

The meaning of —, ±, + and ++ are same as shown in Table 5

Table 11

Sensitivity test of YOSHIDA sarcoma to several anticancer agents

agents \ days	7 T	10T	15T	25T	result
control	+	+	++	++	—
Mitomycin C	—	+	++	++	effective
Tespamin	—	+	++	++	effective
Merphyrin	+	+	++	++	ineffective

The meaning of —, ±, + and ++ are same as shown in Table 5

Table 12 Sensitivity test of human cancers to several anticancer agents

no.	name	age	sex	clinical diag.	histological diag.	Mit. C	Tesp.	Endo.	Merph.	note *
1	T. S.	51	f	stomach-ca.	gallert-ca.	−	+			3W after transpl. tumor cells (±)
2	K. H.	54	f	stomach-ca.	adeno-ca.	−	−			3W after transpl. tumor cells (±)
3	U. S.	61	f	siomach-ca.	skirrlus	+	−			3W after transpl. tumor cells (±)
4	H. T.	43	m	stomach-ca.	ca. simplex		+			3W after transpl. tumor cells (+)
5	K. R.	69	m	stomach-ca,	adeno-ca.	−	−			3W after transpl. tumor cells (+)
6	T. K.	54	m	stomach-ca.	gallert-ca.	−	+			3W after transpl. tumor cells (+)
7	K. Y.	48	f	stomach-ca.	adeno-ca.	−		+		3W after transpl. tumor cells (±)
8	K. K.	32	f	stomach-ca.	adeno-ca.	−		−		2W after transpl. tumor cells (+)
9	N. S.	53	m	stomach-ca.	ca. simplex	−		−		1W after transpl. tumor cells (+)
10	O. T.	54	f	stomach-ca.	adeno-ca.	+		−		1W after transpl. tumor cells (+)
11	K. K.	25	m	retroperitoneal tumor	metastatic seminoma	−	+		+	1W after transpl. tumor cells (+)

+ : effective
− : ineffective

* histological finding of content of the control chamber at the latest experiment of a patient
± : degenerative tumor cells detectable
+ : tumor cells detectable

cells grew as similarly as those in control chambers. Behavior of tumor cells in control chambers described in the note in Table 12 indicates, as mentioned in the chapter of autotransplantation, that autotransplants of human cancers were generally degenerative 3 weeks after transplantation.

Discussion and Conclusion

SANDISON[36,37] had first reported the chamber method for cellular studies. Then, CLARK et al.[9] BICEGLIE[6], EBERT et al.[13] and WILLIAMS[50] reported the same methods. ALGIRE et al.[1,2,4,35] SHELTON et al.[28], AMOS et al.[5], STURGIS et al.[41,42], GABOUREL et al.[16] BROOKS et al.,[8] and many others studied especially on behavior of tumor cells and immunology, by use of diffusion chambers with millipore filter membrane which did not permit direct contact between host cells and target cells in chambers. Following Algire et al., improvement of diffusion chamber technique has been made aiming at its certainty, simplicity and rapidness. To keep continuous perfect adhesion of filter, about 5% acrilite acetic acid solution was used as an adhesive. Autoclave sterilization was better than that by alcohol and formaline gas. During the course of autoclave sterilization, water and steam soluble obnoxious substances were driven away and the chambers were easily dried up and could be used any time without washing in a solution of saline or RINGER's as needed after chemical sterilization. In addition, in the cases of alcohol sterilized chambers, contamination of contents was frequently observed.

Concerning the methods of making cell suspensions from solid tumors, many investigators[7)37)39)] have reported that most important would be how many viable cells could be collected. In obtaining many viable cells from the solid tumors, composed of cells having strong adhesiveness, one could not but be satisfied with coarse cell suspension containing large cell clumps.

The cause of the initial drop in cell count is not elucidated yet as AMOS and WAKEFIELD[5)] stated. However, the following points would have to be considered ; 1) promotion of cell degeneration by time consuming chamber procedures, ii) interruption of route of nutrients by cutting off the cellular connection, iii) humoral resistance suggested by ALGIRE et al.[35)], i. e. hyperimmune circulating antibodies, although humoral factors have heen generally neglected in homograft reaction[28)], iv) disturbance of exchange of nutrients through the filter membrane by the fibrous membrane formed outside the filter. In regard to the first point, the cells were observed to undergo remarkable degeneration a little while after being separated from animals.

Differences of behavior of various tumor cells in chambers were observed. For example, the growth rate of cells was variable according to tumors from which the cells were taken. These differences might partially be due to histoincompatibility between the host and target cells as SNELL[10)] had pointed out.

The morphological changes of tumor cells in chamber might partially be explained as the consequence of grouping cell-growth on the filter which provided a foothold to cells as in cell culture in vitro.

In regard to conditioning it was indicated by many investigators that X-ray irradiation destroyed Properdin and raised susceptibility of the host to tumor transplantion. If conditioning would make heterotransplantation easier, it would be very contributable for tumor investigation. However, an attempt to promote cell growth in the chambers inserted in heterologous animals by conditioning was not successful.

WADE[48)] had first reported destructive action of lymph nodes to homografts in the experiment using dogs in 1908, and ALGIRE et al.[2)] presumed the interaction between target cells and target immunized cells. At present, immune reaction to homograft is considered to be due to cell fixing antibodies which are expected to locate in the lymph nodes. Taking this into consideration, regression or slower growth of original tumors in situ might be due to cell fixing antibodies which are probably contained in lymph nodes. GRILE[18)] emphasized that the significance of lymph nodes would be related to a barrier to cancer invasion, and excision of lymph nodes not yet invaded would rather promote advancement of cancer. On the other hand, MEDAWAR[27)] reported that lymphocytes had not destroyed the tumor cells in vitro. From the histopathological points of view, regional lymph nodes were in a state ready for cancer invasion and had not histoimmunological protecting function against cancer as found by OTA[34)].

Anticancer agents selected by screening test in the spectrum of experimental tumors are not necessarily effective in clinical application. Some of the reasons are as follows ; i) test tumors might resemble each other both in genetics and in susceptibility for anticancer agents, ii) some tumors even of the same genotype and derived from the same organ by just the same artificial stimulation, for example ascites hepatomas, are known

not to have always common effective agents, iii) even the same tumor can react to a certain agent in a different way, when it is treated by a different method of administration. For instance, Merphyrin was not effective for the EHRLICH ascites carcinoma with any method of administration as well as for the EHRLICH solid carcinoma by SUGIURA's method, whereas it is effective for the latter when administered 6 days after transplantation as shown by MIZUNO[31].

It may be said that an evaluation of an effective agent is most correctly done by direct administration to the tumor bearing animal itself. In order to establish a convenient method for selecting effective agents for human cancer, many sensitivity tests have been tried. In this report, taking advantage of participation of abdominal fluid in the test process, sensitivity test by use of diffusion chamber technique were investigated. Even in this method, however, we can not but recognize that the tumor cells to be tested were still in abnormal circumstances.

Of the effects of the agents examined accoring to our method, the following points were worthy of note ; i) Merphyrin was unexpectedly effective for a few tumor cells. Merphyrin was originally made by IIJIMA et al.[20] in an attempt to raise the resistivity of patients to malignant diseases. However, the effect is generally thought not to be constant when administered to animals, and ii) in one of our experiments, temporary growth promoting phenomenon was noticed. It might be said that in a wide sense this phenomenon resembles the adverse effect which was reported by KONDO et al[26].

The purpose of the sensitivity test is to find an agent which is expected to be the most effective to an individual patient. In order to evaluate the usefulness of diffusion chamber technique as a sensitivity test, the preliminary examination was done by use of experimental tumors. The effectiveness of agents tested by this technique was parallel to those of many other experimental therapy of tumors. This might indicate that there would be the same parallelism when the diffusion chamber technique is applied clinically as a sensitivity test. Adverse effect is supposed to take place according to dosage of agents. 2mg/kg of Mitomycin C or 5mg/kg of Tespamin was administered intramuscularly to dd mice with BASHFORD Carcinoma 63 in the chamber daily for the period of 7 days following SUGIURA's method. When such a large dose of agents, as in SUGIURA's method were administered, however, adverse effects were not observed. In other cases in which 0.08mg/kg of Mitomycin C and 0.2mg/kg of Tespamin were administered, no remarkable effects were shown. Consequently it is clear that the doses of anticancer agents influenced the results. The same may be true of "false negative" cases.

Many investigators[10,11,12,14,24,30,33,51,53] reported the various methods of sensitivity tests for human cancers. Clinical sensitivity test to select most effective agents for an individual patient, requires simplicity, rapidness and inexpensiveness in the practice of screening as mentioned by KAJIWARA[24].

The reason for using coarse cell suspensions in clinical application was first in its simplicity in chamber procedures, and second in easier contact with and better nutrition of individual cancer cells than in case of using a larger tumor block. As histological examinations of the contents of chambers were done after one week or so, it was difficult to determine whether or not the degeneration of human cancer cells observed in the cham-

bers at that time would be followed by true cell growth. Here, in order to obtain accurate results with the chamber method, importance of acquiring viable tumor cells as many as possible should be emphasized again. While in I. N. K. method, the indicator of which is tissue activity, accurate quantity and sufficient viability of the specimen are both necessary, in the chamber method, only viability is required.

At present time, there is not enough data to discuss the correlation between the results of chamber technique and actual clinical effects of anticancer agents. In regard to human cancer, WRIGHT[51] showed the relative correlation between clinical and tissue culture response to chemotherapeutics. In his report, the rate of close correlation was 65 percent (26 cases) in 40 cases. In order to get good results in clinical sensitivity tests, it is indispensable that live tumor cells are examined with an anticancer agent. As the tumor cells are sometimes ready to become degenerative within a short time, the results obtained in general cultural methods might present "false negatives". This is a common weak point of the current sensitivity tests. In the chamber method, if the tumor cells would undergo progressive degeneration, the results obtained might be similar to that reported by WRIGHT. In his method the cells tested underwent degeneration. As the tumor cells in the chamber method, however, live longer with peritoneal fluid in vivo, it may be possible to get higher correlation to clinical responses. However, even by use of the chamber technique we can not expect to examine the agents in the same way as the clinical ones.

Consequently, it may be said that an agent selected by use of the chamber method is not necessarily the most effective therapeutic but at least one of several effective therapeutics. Any way, our chamber method presented in this paper may be a promising approach to the perfect one.

SUMMARY

1. For the purpose of clinical application as a sensitivity test, diffusion chamber method was studied.

2. Host reaction, passage of abdominal fluid and passage of host and target cells through the filter were examined by use of the diffusion chamber.

3. Various tumor cells inserted in the chamber in vivo indicated long survival and cell growth following the initial drop. Differences in behavior of tumor cells might be in accordance to their viability. A few cells indicated morphological changes.

4. Heterografts (YOSHIDA sarcoma, EHRLICH ascites carcinoma and MC/5) in the chamber in hosts (dd strain mice and Wistar strain rat) conditioned by X-ray irradiation and predonisolone injection, survived for a longer period of time but did not grow successively.

5. Homogenate of regional lymph nodes inserted in the chamber in vivo together with original tumor cells did not indicate a histoimmunological influence on tumor cells.

6. Considering the fact of long survival and growth of target cells in chambers, a sensitivity test was devised to evaluate the effects of antitumor agents with participation of peritoneal fluid. The results in various experimental tumors were generally parallel to the results of other therapeutic experiments.

7. In the results of the clinical sensitivity test, the rate of effectiveness of Mitomycin C and alkylating agents were respectively 22% and 40% in 10 cases of gastric cancer.

Generally, in cultural sensitivity test, "false negatives" have to be considered and investigated further.

I wish to express my sincere gratitude to Professor CHISATO ARAKI who has been giving me kindest advice and direction and to Assistant Professor IKUZO YOKOYAMA who has been offering a cordial guidance throughout the course of the work. I am also very grateful to Dr. KEISEI TANAKA for his cooperation in X-ray irradiation.

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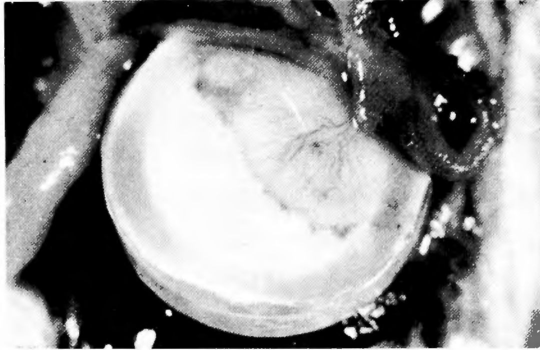

Photo. 1 Vascularisation 30 days after insertion of the chamler

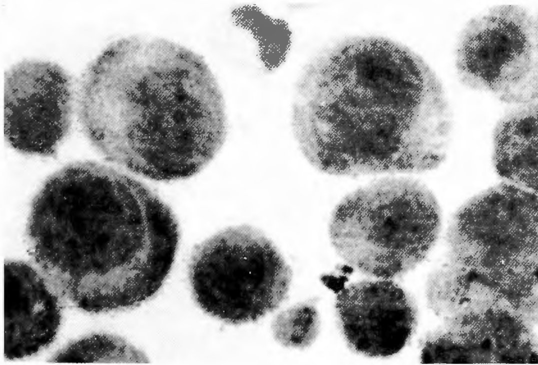

Photo. 2 MH 134 ascites hepatoma 1200×

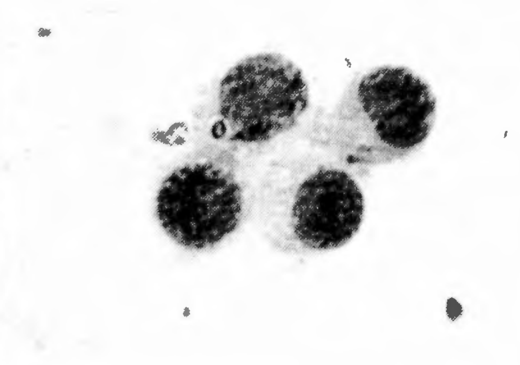

Photo. 3 MH 134 15 days after transplantation 1200×

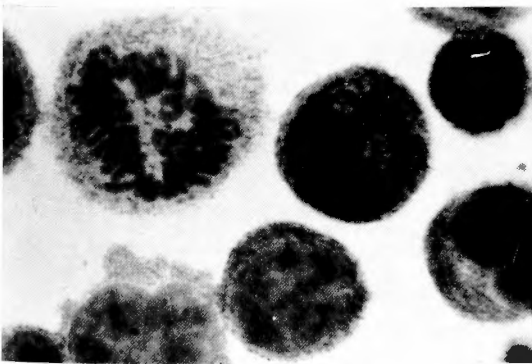

Photo. 4 EHRLICH ascites carcinoma 1200×

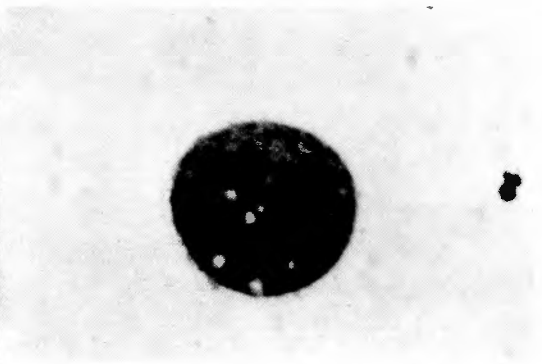

Photo. 5 EHRLICH ascites carcinoma 10 days after transplantation 1200×

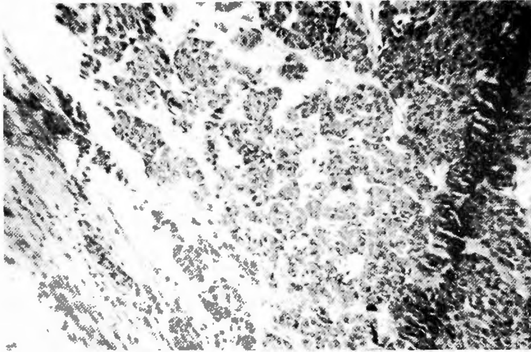

Photo. 6 MC/5 histological finding 100×

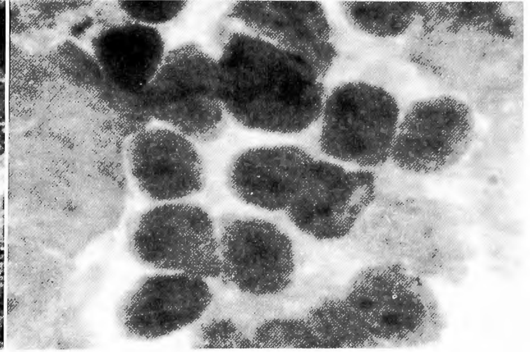

Photo. 7 MC/5 mechanically crushed tumor cells 1200×

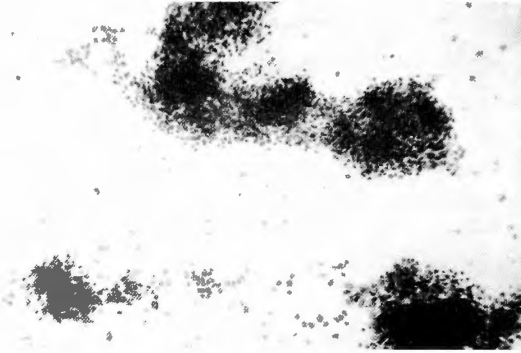

Photo. 8 MC/5 10 days after transplantation 100×

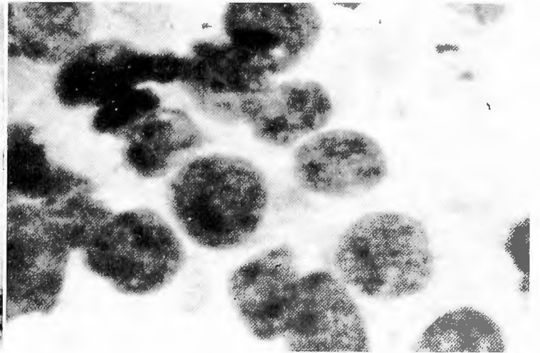

Photo. 9 MC/5 10 days after transplantation 1200×

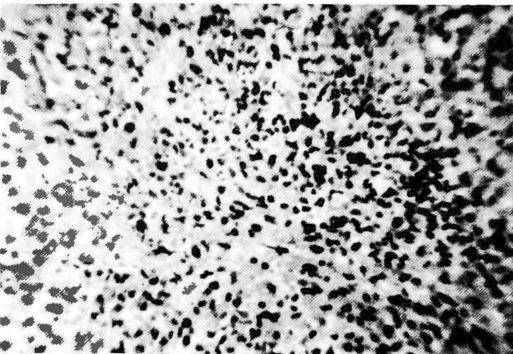

Photo. 10 NF Sarcoma 100×

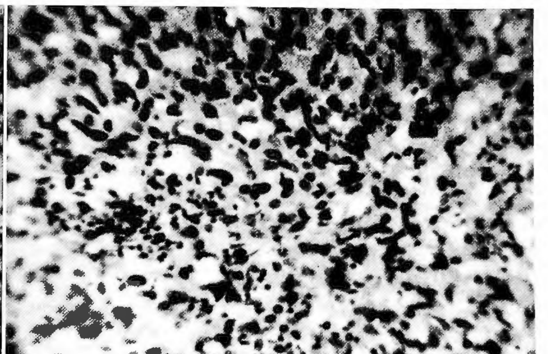

Photo. 11 NF Sarcoma 15 days after transplantation 100×

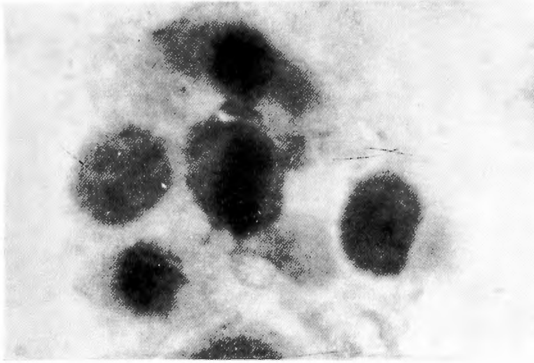

Photo. 12 BASHFORD carcinoma 63 mechanically crushed tumor cells 1200×

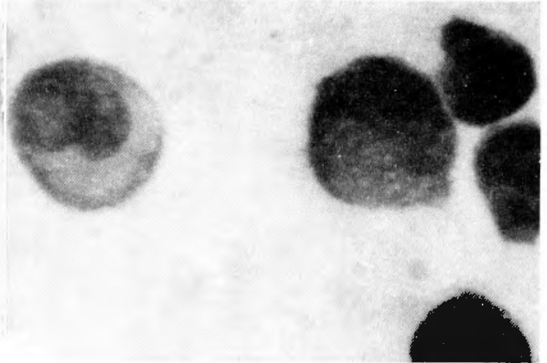

Photo. 13 BASHFORD carcinoma 63 10 days after transplantation 1200×

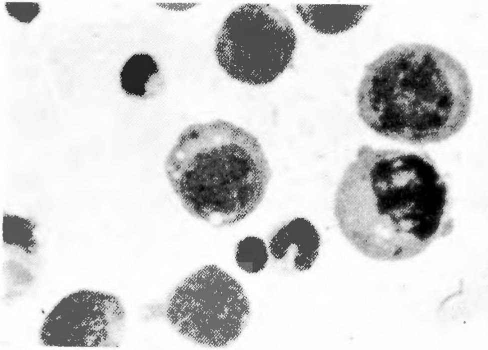

Photo. 14 Sarcoma 180 1200×

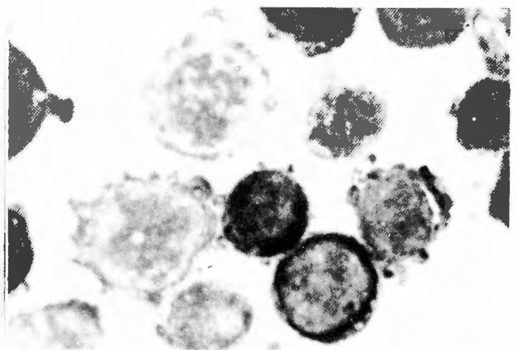

Photo. 15 Sarcoma 180 20 days after transplantation 1200×

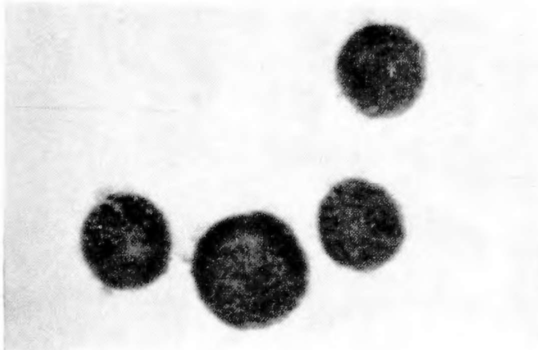

Photo. 16 YOSHIDA sarcoma 1200×

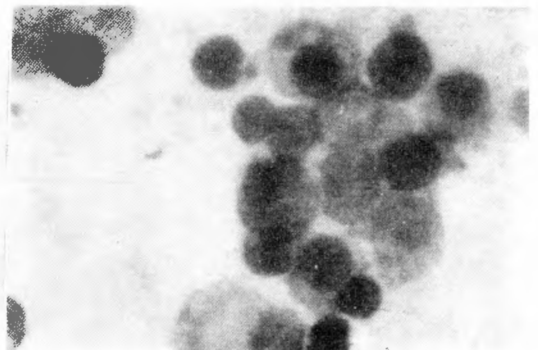

Photo. 17 YOSHIDA sarcoma 15 days after transplantation 1200×

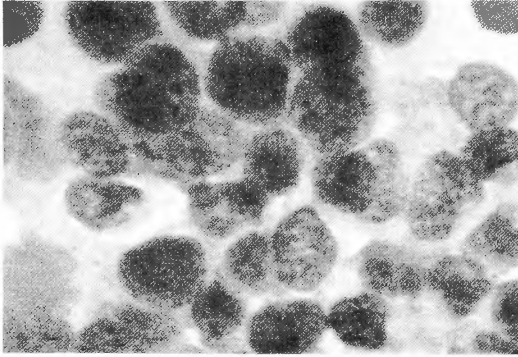

Photo. 18 Human gastric cancer (undifferenciated carcinoma) mechanically crushed tumor cells 1200×

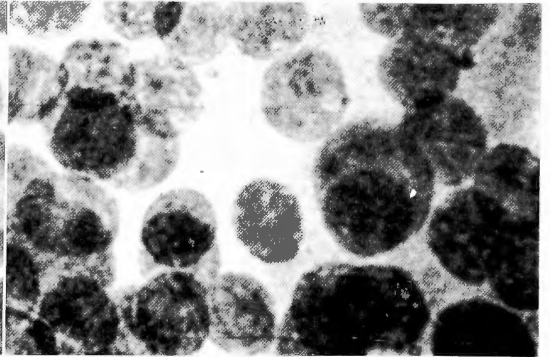

Photo. 19 Human gastric cancer 21 days after transplantation 1200×

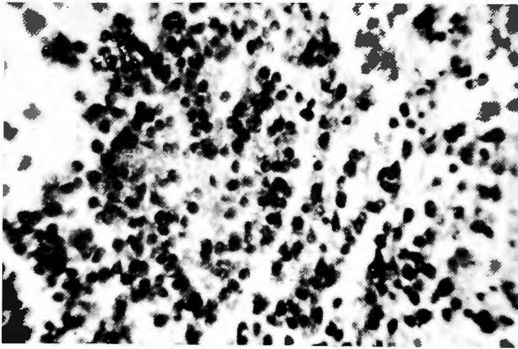

Photo. 20 Metastatic seminoma 100×

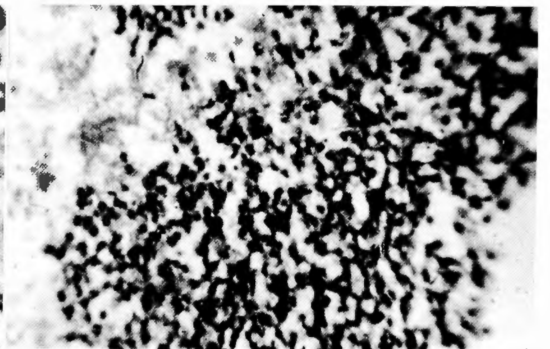

Photo. 21 Metastatic seminoma 21 days after transplantation 100×

和文抄録

癌の治療面に於ける diffusion chamber 法の臨床応用

京都大学医学部外科学教室第1講座（指導：荒木千里教授）

伏 木 信 夫

(1) 癌の治療面に於ける臨床応用を目的として，Algire の原法に準じ diffusion chamber を作製した．この chamber を用い，これを正常動物及び担癌動物の腹腔内に挿入した場合の宿主反応，腹水の経時的浸透率及び宿主細胞，tarrget cells の chamber 内外への漏出入の有無等の諸点を検討した．

(2) MH 134 腹水肝癌，Ehrlich 腹水癌，Sarcoma 180，吉田肉腫，MC/4,5，NF Sarcoma，Bashford carcinoma 63，及び人癌等の各種腫瘍細胞を chamber 内に封入し，実験動物或は患者の腹腔内に挿入すると，autograft,isograft 及び homograft としての腫瘍細胞は initial dropの経過後何れも長期生存乃至は発育増殖の所見を示した．腫瘍によつては，chamber 内に一定時間培養する事によつて細胞学的に形態の変化を生ずるものも若干認められた．

(3) レントゲン，プレドニソロンで conditioning を行なつた dd 系マウス及び Wistar 系ラットの腹腔内へ，吉田肉腫及び Ehrlich 腹水癌，MC/4,5 を封入した chamber を挿入すると，chamber 内腫瘍細胞の比較的著明な life prolongation がみられたが，積極的発育増殖の成績を得る事は出来なかつた．

(4) 癌患者の領域淋巴節の組織免疫学的役割を検討するため，その homogenate を原発巣腫瘍細胞と一定の割合に混合して chamber 内に封入し，組織学的に検索したが陰性の成績を得た．

(5) 腹腔内に挿入した chamber 内に於ける各種腫瘍細胞が長期生存乃至は発育増殖する事実を応用し，体液の関与を含む場に於ける各種制癌剤の sensitivity test を行なつたが，本法による判定成績は実験動物腫瘍に於ては諸家の治療実験の成績と略平行関係の成立する事が試められ，sensitivity test としての本法の有用性が示された．結果の判定には7乃至10日前後が適当と考えられた．

(6) 人癌特に胃癌に就いて sensitivity test を行なつた結果，胃癌10例中，マイトマイシンC：22%，アルキル化剤：40%の有効率を得たので，I.N.K. 法及び其の他の cultural method による sensitivity test の成績と対比し若干の考察を試みた．