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In Vivo Kinetics of Murine Lymphoid Cells: In Relation to Local Adoptive Cancer Immunotherapy

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Summary

In vivo kinetics of lymphoid cells were studied in syngeneic mice using ^{51}Cr labelling. The long-term cultured lymphoid cells showed a poor distribution in recipient mice and were rapidly excreted from the body when they were transferred intravenously, while fresh spleen cells showed good systemic distribution and were well retained. Short-term cultured cells showed an intermediate distribution pattern. But they were hardly detected in the tumor possessing footpads or draining lymph nodes. When these cells were transferred subcutaneously into the footpads, the radioactivity remained there over 48 hours. So, local transfer is essential for adoptive immunotherapy with cultured lymphoid cells.

Introduction

We have been conducting ongoing studies on cancer immunotherapy with adoptive transfer of lymphocytes, and recently reported promising effects of this therapy in the syngeneic tumor-mice system. The spleen cells which were obtained from tumor-bearing mice, educated and expanded by mixed lymphocyte tumor cell culture (MLTC) and T cell growth factor (TCGF), eradicated subcutaneously (sc) transplanted tumor when they were transferred locally[13,14]. But in our therapy model, no effects were observed with intravenous (iv) administration of cultured cells.

Many trials for cancer therapy with adoptive transfer of in vitro educated lymphoid cells have been reported[2,3,22]. Recently some investigators have developed adoptive cancer immunotherapy models with remarkable effects using lymphoid cells cultured and proliferated with TCGF in vitro[4,8,17]. However many of them adopted local injection into the tumor site or intraperitoneal injection against the ascitic form of cancer as the route of administration of

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Abbreviations used: TCGF: T cell growth factor, MLTC: Mixed lymphocyte tumor cell culture, MMC: Mitomycin C, SC42: Shionogi Carcinoma 42, FBS: Fetal bovine serum, HEPES: N-2-Hydroxy-ethyl-piperazine-N'-2-ethane sulfonate, 2ME: 2-Mercaptoethanol, HBSS: Hanks' balanced salt solution, FSC: Fresh spleen cells, DBL: DS blast lymphoid cells

cultured cells, and at the same time, they reported that there was little or no effects by iv administration. In the reports with favorable effects by iv administration, relatively short-term cultured cells were used in a large amount[5,8]. Several reasons were suspected as to why the iv administration of these cytotoxic lymphoid cells was ineffective; The most important being the difference in distributions of these cells in the body between the routes of administration.

In this report, the distributions of fresh spleen cells, short-term cultured lymphoid cells with anti-tumor activity and long-term cultured TCGF-dependent lymphoid cells were studied following iv or local sc transfer with ^{51}Cr labelling.

Materials and Methods

Animal

Inbred 13 to 16 week-old male DS mice, sublined from dd mice[27] were obtained from Aburabi Laboratory of Shionogi Pharm. Co. (Shiga).

Tumor

Shionogi Carcinoma 42 (SC 42) established from spontaneous mammary carcinoma of DS mice[18] was kindly donated by Dr. *Yamaguchi* and maintained serially in DS male mice by sc inoculation. Single tumor cell suspension was prepared by digestion with 0.25% trypsin (Difco, Detroit, Mich.). When 1×10^5 of SC 42 cells were inoculated into the flank or 4×10^5 into the footpad subcutaneously, tumors became palpable 10 to 17 days after inoculation and were lethal in 25 to 60 days. Mice inoculated with 4×10^5 of SC 42 cells into their right hind footpads 10 days previously were designated tumor-bearing mice. When the right leg of mice bearing SC 42 tumor in r. footpad (14 days after inoculation) was amputated, all the mice acquired strong in vivo anti-tumor activity which was capable of rejecting tumor rechallenge (2×10^5) into the flank; these mice were designated immune mice. Before mixed lymphocyte tumor cell culture (MLTC), SC 42 cells were preincubated for 40 min at 37° in RPMI 1640 (Nissui, Tokyo) with 50 μg/ml of Mitomycin C (MMC).

TCGF and Lectin-free TCGF

TCGF used for maintainance and expansion of DS blast lymphoid cells (DBL) was prepared according to the method of *Gillis*[9]. Lectin-free TCGF was prepared according to the method of *Spiess*[24]. Briefly, spleen cells obtained from 8 to 12 week-old CD rats were preincubated with 10 μg/ml Concanavalin A (Con A) (Miles-Yeda, Rehovot, Israel) in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS) (GIBCO, Grand Island, N.Y.), 20 mM N-2-Hydroxyethyl-piperazine-N'-2-ethane-sulfonate (HEPES), 50 μm 2-Mercaptoethanol (2ME) and 100 μg/ml gentamycin (complete culture medium) for 2 hours at 37° in 5% CO_2 in humidified air. Then the activated cells were washed thoroughly with Hanks' balanced salt solution (HBSS) and cultured for 24 hours at a concentration of 2×10^6 cells/ml in the complete medium without the lectin in 5% CO_2 in humidified air. At the end of the culture the supernatant was collected and stored at −20° until use.

Fresh Spleen Cells (FSC)

Spleens were obtained aseptically from normal DS mice. Single cell suspensions were prepared with passage through a #100 stainless steel mesh. Erythrocytes were lysed with the treatment of 0.83% NH_4Cl in Tris-buffer for 5 min. Spleen cells were washed with HBSS and suspended in the medium for labelling described below.

When the FSC were obtained from immune mice, they were designated "immune FSC". They have in vivo tumor neutralizing activity[13,14].

8 day MLTC Cells

Spleen cells were obtained from tumor bearing mice and suspended in RPMI 1640 supplemented with 10% FBS. They were incubated for 40 min at 37° in 10 cm plastic dishes (Corning, N.Y.).

Plastic nonadherent cells were collected after gentle agitation, and cultured with the MMC-treated SC 42 cells at a responder to stimulator cell ratio of 10 : 1 with 50% lectin-free TCGF. Cultures were performed in Linbro 2 cm^2 multi well culture plates (Flow lab., Mclean, Va.) at 37° in 5% CO_2 in humidified air. Culture medium for MLTC was RPMI 1640 supplemented with 7% heat-inactivated pooled human AB serum, 20 mM HEPES, 50 μM 2ME and 100 μg/ml gentamycin. In the primary culture, the spleen cells were adjusted to 3.5×10^6/ml and cultured for 5 days. In the secondary culture, the cells were adjusted to 1.5×10^6/ml and cultured for 3 days. At the termination of the culture, cells were collected, washed with HBSS and suspended in the medium for labelling. Anti-tumor activity of these cells have been confirmed in the Winn's assay and local adoptive immunotherapy model[13,14].

DS Blast Lymphoid Cells (DBL)

The tumor-infiltrating lymphoid cells which had been separated from SC 42 tumor mass were established as a cell line and maintained with TCGF for over 6 months in our laboratory. These cells are dependent on TCGF and cannot survive over 24 hr without TCGF. Anti-tumor activity of these cells was not detected in the Winn's assay, however they have slight natural killer (NK) cell-like activity in ^{51}Cr-release assay[12].

^{51}Cr Labelling and Transfer of the Cells

$1–2\times10^8$ of these cells were suspended in 3 ml of RPMI 1640 supplemented with 10% FBS and 20mM HEPES. They were incubated with 200 μCi of $Na_2{}^{51}CrO_4$ (Daiichi RI Lab., Tokyo) for 1 hr at 37° with occasional agitation. At the end of incubation, the cells were washed thoroughly with HBSS and suspended in RPMI 1640 at cell concentrations of 4×10^6 in 0.05 ml for sc injection and 4×10^6 in 0.3 ml for iv injection.

Normal or tumor bearing mice under ether inhalation anesthesia received either sc injection of the labelled cells into the right hind footpad or iv injection through the tail vein.

Killed DBL (kDBL) were prepared from the final suspension of ^{51}Cr labelled DBL by 3 cycles of freezing at −80° and thawing at 40° before transfer.

Counting of Radioactivity

Mice were sacrificed 2, 24 and 48 hr after the transfer of the ^{51}Cr labelled cells. Whole body radioactivity was counted in an animal counter TAC-1 (Tokyo Atomic Ind. Co.). Both hind legs, both inguinal lymph nodes with surrounding adipose tissues, lungs, kidneys, liver and spleen were removed, and their radio-activities were counted in Auto-well Aloca (Aloca, Tokyo). Aliquots of transferred cells were also counted in the same instruments. Each group consisted of three mice. Net injected dose was determined by comparison of radioactivities obtained from the aliquots of transferred cells and whole body activities at 2 hr after transfer, because no remarkable decrease in whole body radioactivity during the first 2 hr after transfer was seen whether the cells were transferred iv or sc.

Results

Distribution of Radioactivity in the Normal Mice Following IV Transfer of the ^{51}Cr Labelled Cells

Normal DS mice were injected with 4×10^6 of ^{51}Cr labelled cells through the tail vein, and radioactivity of the whole body and respective organ i.e. lungs, kidneys, liver and spleen was measured at various intervals (Fig. 1). In the mice to which fresh normal spleen cells (FSC) were transferred, liver and spleen radioactivities were 30% and 20% respectively after 2 hr. The whole body radioactivity was about 80% at 24 hr and 48 hr after transfer and the levels in the liver and in the spleen were also 30% at these time intervals. Only 14% was found in the lung at 2 hr. On the other hand, in the mice to which long-term cultured DS blast lymphoid cells

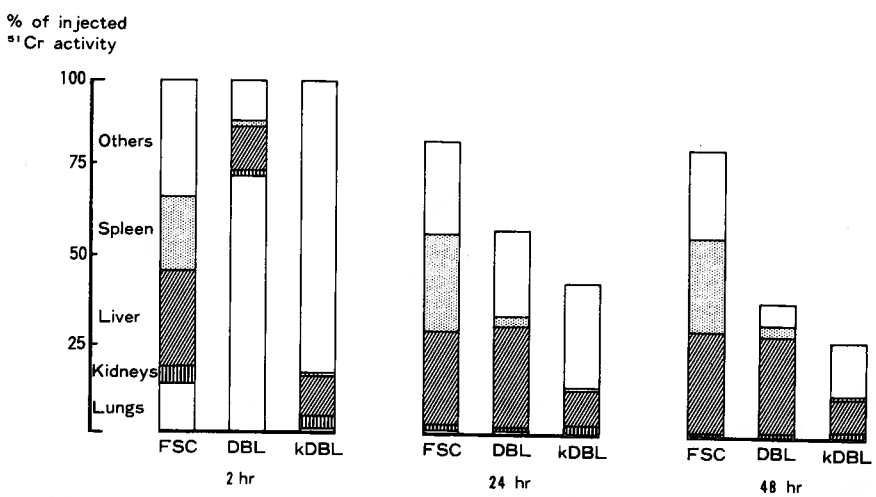

Fig. 1. Distribution of radioactivity in the normal mice following iv transfer of the ^{51}Cr labelled cells
Normal mice were sacrificed 2, 24 and 48hr after iv transfer of the ^{51}Cr labelled cells, and radioactivity of whole body, spleen, liver, kidneys and lungs was measured. "Others" indicates the difference between the radioactivity of whole body and the sum of each organs.
FSC: Fresh spleen cells, DBL: DS blast lymphoid cells, kDBL: killed DBL.

(DBL) were transferred, lung radioactivity was 70% at 2 hr. At 24 hr, less than 1% was seen in the lung whereas liver radioactivity increased from 14% to 30%, while only 3% was found in the spleen. The whole body radioactivity rapidly decreased. When the killed DBL (kDBL) were injected intravenously, less than 1% of transferred radioactivity was found in the lung at 2 hr and only 10% in the liver; About 85% of the radioactivity belonged to the "others" category. The liver radioactivity did not increase thereafter.

8d MLTC cells showed an intermediate pattern of distribution between FSC and DBL when they were transferred iv into normal recipient mice (Fig. 2). At 2 hr the lung radioactivity was 30% and that in the liver 26%. Similar distribution in the both organs was observed at 24 hr and 48 hr in the mice to which FSC, DBL or 8d MLTC cells were transferred. In the mice receiving 8d MLTC cells, spleen radioactivity was 10–15% throughout the 48-hr period, a pattern intermediate between DBL (2–3%) and FSC (20–30%).

Different In Vivo Kinetics of the Labelled Cells According to the Route of Transfer

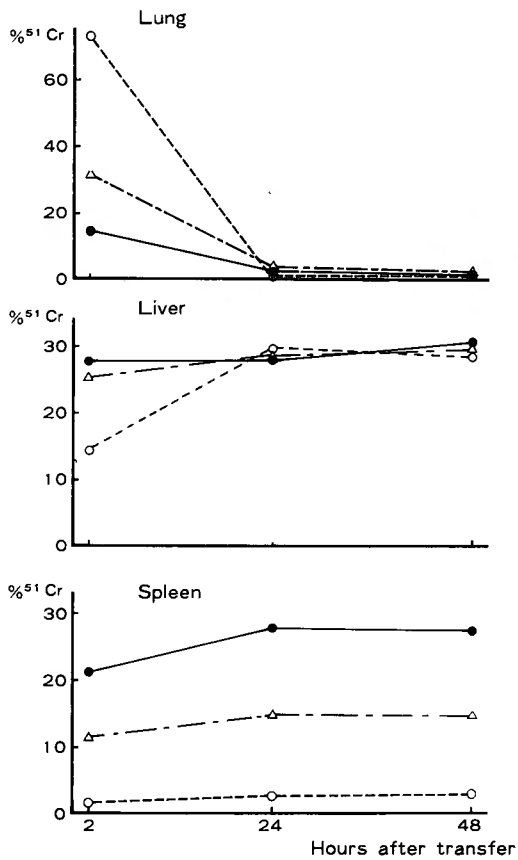

Fig. 2. Change in radioactivity of respective organs following iv transfer of the ^{51}Cr labelled cells
Normal mice were iv injected 4×10^6 of ^{51}Cr labelled FSC (●—●), 8d MLTC cells (△-·-△) or DBL (○···○). The radioactivity of the lungs, liver and spleen was measured at various intervals.

When the ^{51}Cr labelled FSC were transferred subcutaneously into the right hind footpad, the radioactivity of the whole body rapidly decreased compared with iv injection (Fig. 3). DBL showed a similar pattern with both routes of transfer. However the radioactivity recovered from the footpad where the cells were transferred was well retained; For FSC, over 70% at 2 hr and 20% at 48 hr after transfer were recovered from this region. More than 30% at 2 hr and around 10% thereafter were recovered in the case of DBL. Hardly any radioactivity was detected in the footpad when either cells were transferred iv. At the same time, when the cells were injected sc, almost no radioactivity was detected in the spleen (Table I), and about 2 to 5% of radioactivity was recovered from the liver and kidney irrespective of the kind of transferred cells.

In Vivo Kinetics of the Labelled Cells in the Syngeneic Tumor Bearing Mice

The tumor bearing mice to which 4×10^5 of SC 42 cells had been inoculated in their right hind footpad 10 days before were administered ^{51}Cr labelled immune FSC or 8d MLTC cells. The radioactivity of spleen, bilateral inguinal lymph nodes with surrounding adipose tissues and r. hind leg was examined (Table II). When the cells were transferred iv, the radioactivity of r. footpad was about 0.6% in the mice injected with immune FSC and 0.4–0.5% in those receiving

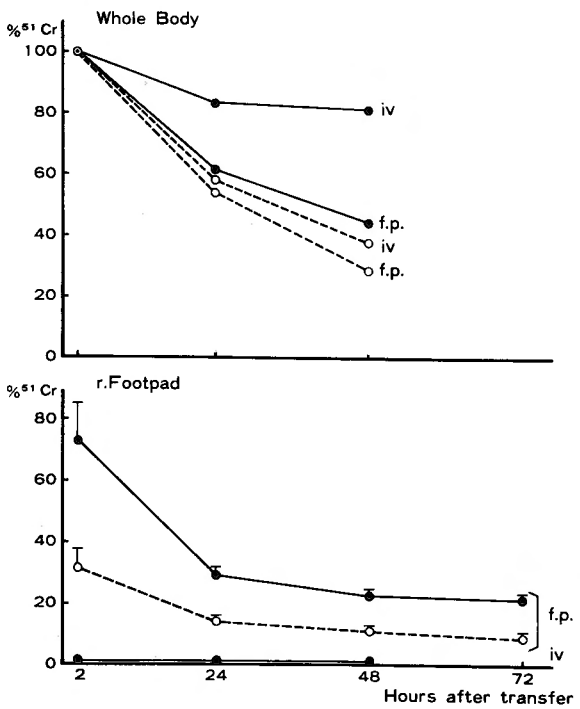

Fig. 3. Change in radioactivity following transfer of the ^{51}Cr labelled cells
Normal mice were injested with 4×10^6 of ^{51}Cr labelled FSC (●—●) or DBL (○---○) sc into the right hind footpad (f. p.) or iv. The radioactivity of the whole body and the r. footpad was measured at various intervals. Longitudinal bar indicates SD.

Table I. Distribution of ^{51}Cr Labelled DBL and FSC in Normal DS Mice

	Mean % of injected radioactivity±SD			
	intravenous injection		footpad injection (a)	
	DBL	FSC	DBL	FSC
Spleen				
2hr	1.8±0.1	20.6±5.5	0.12±0.04	0.22±0.03
24hr	2.8±0.3	27.6±0.9	0.08±0.002	0.59±0.19
48hr	3.0±0.2	26.7±2.8	0.08±0.008	0.52±0.12
Liver				
2hr	14.4±0.6	27.1±0.9	2.3±0.3	2.8±0.7
24hr	28.5±3.3	26.3±1.5	1.6±0.05	4.8±0.3
48hr	27.2±0.9	28.7±0.3	1.4±0.2	3.3±0.2
Kidney				
2hr	1.6±0.09	4.5±0.2	2.8±0.3	2.6±0.9
24hr	1.7±0.08	2.1±0.3	2.2±0.1	5.2±0.5
48hr	1.2±0.06	1.6±0.4	1.5±0.1	4.3±0.6

a) Cells were injected subcutaneously into the right hind footpad.

8d MLTC cells. The radioactivity of inguinal lymph nodes on either side was about 0.3% in the mice transferred either kind of cells. Though both immune FSC and 8d MLTC cells have in vivo tumor neutralizing activity[13,14], no specific accumulations of them in the tumor nest or draining lymph nodes were observed.

When administered sc into the r. footpad, the radioactivity of r. inguinal lymph nodes was less than 0.1% with the same amount being observed in l. inguinal lymph nodes in contrast to the high amount of r. footpad.

In addition, when the cells were transferred iv, preliminary experiment (data not shown) revealed that there was no difference between the radioactivity of the footpad of right (tumor bearing side) and left.

Discussion

There have been many reports on in vivo kinetics of fresh or short-term cultured lymphoid cells transferred intravenously[1,6,7,11,15,19,20,23,28], but only few reports using lymphoid cells cultured with TCGF[16,25].

In present experiments, the liver to spleen (L/S) ratio of radioactivity at 24 hr after iv injection was about 1 for uncultured cells and about 10 for DBL. More than 70% of transferred DBL was temporarily trapped in the lung at 2 hr after iv transfer. On the other hand, only 14% of FSC was found in the lung at 2 hr. These findings were identical to those reported by *Lotze* et al.[16]. *Rouse*[23] also observed by use of autoradiography that, when thymocytes cultured for 6 days were transferred iv, about 20% of them were trapped in the alveolar wall of the lung at 4 hr after transfer but not at 24 hr. Long-term cultured cells with TCGF are blastic and very large, and their surface condition would differ from the uncultured lymphoid cells. Thus, in

Table II. Distribution of ^{51}Cr Labelled 8d-MLTC Cells and Immune FSC in SC 42 Bearing DS Mice (a)

	Mean % of injected radioactivity±SD			
	intravenous injection		footpad injection (b)	
	8d-MLTC cells (c)	immune FSC (d)	8d-MLTC cells	immune FSC
Spleen				
2hr	17.4±0.9	30.9±1.1	0.07±0.005	0.05±0.006
24hr	22.7±0.7	31.6±0.8	0.10±0.03	0.11±0.02
48hr	21.4±2.6	28.4±2.8	0.10±0.02	0.15±0.03
r. Footpad				
2hr	0.37±0.19	0.77±0.32	63.0±5.6	69.4±7.7
24hr	0.54±0.07	0.66±0.07	34.4±9.1	42.6±5.8
48hr	0.44±0.05	0.54±0.13	20.2±3.1	31.1±3.8
r. Ing. LN (e)				
2hr	0.24±0.05	0.20±0.02	0.08±0.02	0.06±0.002
24hr	0.21±0.04	0.26±0.03	0.07±0.01	0.09±0.04
48hr	0.20±0.02	0.21±0.06	0.05±0.02	0.12±0.10
l. Ing. N				
2hr	0.21±0.01	0.21±0.02	0.09±0.05	0.07±0.01
24hr	0.22±0.01	0.25±0.06	0.06±0.003	0.06±0.01
48hr	0.17±0.02	0.21±0.03	0.04±0.02	0.07±0.01

a) Mice were inoculated 4×10^5 of SC 42 cells into their right hind footpad 10 days previously.
b) Cells were injected subcutaneously into the right hind footpad.
c) Spleen cells obtained from SC 42 bearing mice were cultured for 8 days with MMC treated SC 42 cells prior to transfer.
d) Fresh spleen cells were obtained from SC 42 immune mice. (see Materials and Methods)
e) Inguinal Lymph Nodes

accordance with our finding that at 24 hr little activity was recovered from the spleen and re-accumulation occurred in the liver, some kind of selection or readjustment might take place during temporary trapping in the lung as Rouse had speculated. When the DBL were killed before transfer, the fate of the larger part of the injected radioactivity could not be detected. However considering the facts that the larger part of the distribution pattern of kDBL expressed here reflects the pattern of free ^{51}Cr released from the dead cells and that re-uptake by host tissues of once released ^{51}Cr is almost negligible[1], viable DBL may have reaccumulated in the liver after 24 hr.

In the case of 8d MLTC cells, the L/S ratio at 24 hr after iv transfer was 2.3, and trapping in the lung at 2 hr was about 31%, showing intermediate in vivo kinetic pattern between that of DBL and fresh spleen cells. Therefore the ability of transferred cells to circulate systematically is reduced with longer culture periods.

When lymphoid cells are adoptively transferred into cancer patients, there may be at least two ways they can exert their anti-tumor activity. One is to settle in the spleen or draining lymph nodes of recipient as memory or helper population, and to induce or recruit specific effector

cells from the host which attack tumor cells; the other is to attack tumor cells directly as an effector population.

The first method is thought to be much more beneficial to cancer patients since the effects would be long-lasting, but it requires redistribution of viable cells into the spleen or other lymphoid tissues following iv transfer. This may be possible in the case of uncultured or short-term cultured cells. Actually long-lived memory cells were observed in the lymphoid tissues of recipient[7,11]. *Fernandez-Cruz*[8] reported in his therapy model that the effective iv transferred cells consisted mainly of helper subset. However it would be difficult at present for long-term cultured cells with TCGF to act as memory or helper cells because of some limitations. Firstly, in vivo environment is very unfavorable for TCGF-dependent cells since, as *Hardt* reported, there is a TCGF inhibitor in the normal mouse serum[10]; and that the disappearance rate of exogenous TCGF from the blood after iv injection is very rapid[21]. Secondly, as we observed, long-term cultured cells recirculate poorly into the spleen or other lymphoid tissues. Thirdly, because long-term cultured cells with TCGF or their cloned cells have acquired complete TCGF dependence, they can hardly survive for over 24 hr in vitro in a TCGF-free medium, though there is some evidence on the survival of TCGF-dependent cells in vivo environment without TCGF[25]. Thus, at present, the direct cytotoxic approach using long-term cultured cells appears more suitable in adoptive immunotherapy.

In the present study, when either kind of cells were injected subcutaneously into the tumor site, the radioactivity of the whole body rapidly decreased. Moreover only 30% of the injected radioactivity was recovered from the injected site 2 hr after sc transfer of DBL. These results may indicate that many of the transferred cells were devitalized shortly after their sc transfer especially in the case of long-term cultured cells with TCGF. But a larger number of cells can be expected to remain compared with iv injection, as demonstrated by our findings that about 11% of the injected radioactivity still remained in the injected site 48 hr after local injection of DBL, though their viability at that time was not clear. Considering the fact that direct contact with target cells is required for the transferred cells to act as a direct effector against tumor, this difference in cellular accumulation at the tumor site between local and iv injection seems to play a definitive role in the therapeutic effect by a definite number of transferred cells. These results agree with the discouraging findings upon iv transfer of cells observed by many investigators. *Winn* reported that immune lymphoid cells administered sc with the tumor cells were from 5 to 100 times more effective in transferring immunity than when administered intraperitoneally and that immune cells administered sc on the side opposite to that of the tumor were about 50% as effective as cells administered ip[26].

Some investigators had reported increased localization of iv transferred lymphoid cells in the antigen-stimulated draining lymph nodes[6,7,28]. However in our experiments involving 8d MLTC cells or immune FSC injected iv into the tumor-bearing mice, no specific accumulation in the tumor nest or draining lymph nodes was observed despite the anti-tumor activity. To detect the specific accumulation of transferred cells, if present, we may have to devise a more elaborate method considering the relatively small population having actual specificity within the trans-

ferred cells and low antigenicity of the tumor.

It is interesting that much lower radioactivity than the case of iv injection was detected in the draining lymph nodes with surrounding adipose tissues when the fresh cells or cultured cells were injected sc into the tumor site. This indicates that when the cells are transferred locally sc, the population remaining in the draining lymph nodes is almost negligible in the course of diffusion to the systemic circulation. From these results and the fact that little radioactivity is observed in the spleen, locally administered cultured cells appear to exert their anti-tumor activity mainly as direct effector against tumor cells at the tumor site. However from the fact that some of the mice, which survived in the Winn's assay or therapy model using the local administration of cultured cells, acquired strong anti-tumor immunity (our unpublished observation), local adoptive immunotherapy also may induce anti-tumor immunity of the recipient by some unknown mechanism.

Recently, *Cheever* reported that anti-tumor activity of adoptively transferred cells could be boosted with repeated administration of highly concentrated TCGF[5]. These results are promising for the adoptive immunotherapy since they indicate that the viability of transferred cells may be prolonged and their redistribution may be promoted.

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和文抄録

マウスリンパ球の生体内動態：癌局所受動免疫療法と関連して

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^{51}Cr 標識法を用いて，DS マウスにおける培養リンパ球または新鮮脾細胞の生体内動態を検討した．静脈内投与した場合，同系腫瘍 (SC42) 浸潤リンパ球由来で TCGF 依存性を有する長期培養リンパ球株は，全身分布状態悪く，速やかに体内から排出されるのに対し，新鮮脾細胞は広く体内に分布し，しかもよく保持されていた．担癌マウスの脾細胞を8日間 TCGF 存在下に MMC 処理腫瘍細胞と混合培養した短期培養リンパ球は，上記二者の中間の動態を示した．これらのリンパ球を正常マウスまたは右足蹠担癌マウスの右足蹠に皮下投与すると，その放射活性は48時間以上投与局所に留まるが，脾臓や所属リンパ節にはほとんど認められなかった．腫瘍免疫マウスから得られた新鮮脾細胞や短期培養リンパ球は抗腫瘍活性を持つが，これらのリンパ球を静脈内投与しても，担癌局所や所属リンパ節にはほとんど集積を確認できなかった．以上より培養リンパ球を用いた特異的受動免疫療法を行なう場合には局所投与が肝要であることが示唆される．