主論文

Generation of Therapeutic T Lymphocytes from Tumor-bearing Mice by the in vitro Sensitization (IVS): Feasibility of Cryopreservation of Preeffector Cells, Effector IVS Cells and Long Term Expansion of Effector Cells

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In Vitro Sensitization (IVS) 培養法による，担癌宿主からの抗腫瘍エフェクターT細胞の誘導：エフェクター前駆細胞，抗原腫瘍細胞とエフェクターT細胞自身の凍結保存性および，エフェクターT細胞の長期培養に関する研究

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Original Articles

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Summary

Using weakly immunogenic murine MCA 105 and 106 fibrosarcomas, we have previously demonstrated that therapeutic cells could be generated by an in vitro sensitization (IVS) culture with which lymphocytes from tumor-bearing mice were co-cultured with viable autologous tumor cells in the presence of low dose of recombinant interleukin-2 (RIL-2). Adoptive transfer of the IVS cells could effectively mediate not only the regression of established visceral metastasis, but also cure and result in long term immune to autologous tumor challenge in treated mice. Applying this IVS culture system clinically, it would be ideal to control the initiation of the IVS culture, especially when combining with other treatment, such as chemotherapy and irradiation. In the present study, feasibility of cryopreservation of responder lymphocytes, stimulator tumor cells before IVS culture, and of the effector IVS cells were evaluated. The data presented here demonstrated that responder lymphoid cells obtained from tumor-bearing mice could be successfully cryopreserved while maintaining their ability to be sensitized. Fresh tumor cells, as well as established tumor cell lines in vitro could be cryopreserved without losing antigenic stimulator potential during IVS culture. Furthermore, the IVS cells generated in the initial 9-day IVS culture could be expanded while maintaining their anti-tumor effect in vivo by periodic antigenic stimulation with fresh or in vitro cultured tumor cells in the presence of RIL-2. Our data suggested that IVS culture could be initiated at optimal time which enables us to combine the adoptive immunotherapy with other therapy in various clinical situations.

Key words : Adoptive immunotherapy, In vitro sensitization, Cryopreservation, Long term expansion of effector cells, Pulmonary metastasis

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Introduction

Recent progress of cellular immunology and molecular biology in cytokine research have enabled us to manipulate immune system for the treatment of cancer. From the first successful large scale clinical trial utilizing lymphokine-activated killer (LAK) cells with recombinant interleukin-2 (RIL-2), adoptive immunotherapy has been established as a significant therapeutic strategy in human cancers[1),2)]. In several animal experiments, adoptive transfer of sensitized T cells has been shown to be highly effective for mediating the regression of established tumors, but successful adoptive immunotherapy required large numbers of properly sensitized, syngeneic effector cells[3)~6)] Recently, we have established an in vitro sensitization (IVS) procedure with which lymphocytes from tumor-bearing mice could be sensitized and expanded to acquire anti-tumor effect in vivo [7),8)] Furthermore, effector cells generated from the IVS procedure could mediate the regression of not only microscopic, but also advanced visceral metastases and subsequently resulted in cure of some mice[9)]. For clinical application of this IVS procedure, preservation of responder lymphocytes and tumor stimulator cells would be crucial for the optimal timing of the initiation of IVS culture in each case. In the present study, we have studied the feasibility of cryopreservation of responder lymphocytes, stimulator tumor cells and IVS effector cells. Furthermore, in vivo and in vitro anti-tumor activity of long term cultured IVS cells was examined to explore the possibility to increase the number of effector cells transferred.

Materials and Methods

Mice Female C57Bl/6J (B6) mice were obtained from Japan CLER Co., Japan. They were maintained in specific pathogen-free conditions and were used at the age of 10 wk or older.

Tumors The MCA 105 tumor is a 3-methylchoranthrene induced fibrosarcoma syngeneic to B6 mice[6)]. The tumor was maintained in vivo in syngeneic mice by serial s. c. transplantation of cryopreserved tumor samples as previously described[6)] and used for the current study in the 3rd to 8th transplantation generation. Single cell suspension was prepared from solid tumors by digestion with constant stirring in 50 m*l* of HBSS (GIBCO, Grand Island, NY, U. S. A.) containing 5 mg of deoxyribonuclease, 50 mg of collagenase, and 100 U of hyaluronidase (Sigma Chemical Co., St. Louis, MO, U. S. A.) for 3 hr at room temperature. The MCA 102 fibrosarcoma also induced with methylcholanthrene served as specificity control.

For initiating solid tumor growth, B6 mice were inoculated in the footpad with 1×10^6 MCA 105 tumor cells in 0.05 m*l* of HBSS. The growth of tumors was evident on day 3 and all mice gradually died from the progressively growing tumor with a median survival time of approximately 25 days.

Lymphoid cell suspensions. Popliteal lymph nodes draining the footpad tumor for 7 to 16 days were removed aseptically. Single cell suspensions were prepared first by teasing with 18 gauge needles followed by pressing mechanically with the blunt end of a plastic syringe plunger in HBSS under sterile conditions. Spleens from tumor-bearing mice and normal B6 mice were similarly prepared and the cell suspensions were filtered through a layer of No. 100 nylon mesh (NYTEX. TETKO Inc., Elmsford, NY, U. S. A.), centrifuged and Red blood cells were lysed by treatment with ammonium chloride-potassium lysing buffer (8.29 g NH_4Cl, 1.0 g $KHCO_3$, and 0.0372 g EDTA/

liter. pH 7.4). The cells were washed twice with HBSS, suspended in culture medium (CM), or cryopreserved for further experiments. CM was composed of RPMI 1640 with 10% heat-inactivated fetal bovine serum, 1 μM sodium pyruvate, 0.1 mM non-essential amino acids, 2 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml tobramycin, 0.5 μg/ml amphotericin B (Fungizone, Bristol-Myers Squibb K. K., Tokyo, Japan) and 5×10^{-5} M 2-ME (Sigma).

For establishing tumor cell lines, 2×10^6 freshly harvested MCA 105 tumor cells were cultured in CM in 75 cm^2 culture flask (No. 25110, Corning Glass Works, Corning, NY, U. S. A.). After 4 to 5 days when tumor cells became confluent, tumor cells were harvested by short incubation with a solution of 0.25% trypsin. Detached tumor cells were washed and resuspended in CM for further culture. When used in IVS, cultured tumor cells were harvested by trypsinization and given 20 Gy irradiation. For irradiation, tumor cells were adjusted to concentrations of 1 or 2×10^7/ml in HBSS and were γ-irradiated at a rate of 2.5 Gy/min.

Cryopreservation of cells. Tumor cells and lymphoid cells were washed 3 times with HBSS and were resuspended in heat-inactivated FCS at the cell concentration of 2 to 4×10^7/ml. After addition of DMSO at the final concentration of 10%, cell suspensions were aliquoted to cryotubes (Serum tube. SUMILON, Tokyo, Japan), placed in −80℃ deep freezer for 18 hrs and then transferred to liquid nitrogen tank for long-term storage. When used, cryopreserved cells were thawed rapidly in 37℃ water bath and washed twice with CM.

IL-2. The human RIL-2 for the current study was kindly supplied by TAKEDA pharmaceutical Co., Osaka, Japan. The biologic and biochemical properties of the IL-2 have been described in detail elsewhere[10)~12)]. Purified material had a specific activity of 1.17×10^7 U/mg protein. The endotoxin level in the purified preparation was less than 0.1 ng/10^6 U of IL-2 as assessed by the standard Limulus assay.

IVS of lymphoid cells. The procedure and optimal culture condition for IVS have been detailed previously[8)]. In the present study, 4×10^5 lymphoid cells were co-cultured with 2×10^5, irradiated (20 Gy) tumor stimulator cells in the presence of RIL-2 (40 U/ml) in 2 ml of CM in 24-well, flat-bottomed tissue culture plates (No. 25820, Corning). The cultures were incubated at 37℃, 5 % CO_2 in a moisturized incubator. The growth of lymphocytes became evident after 5 to 6 days and then cultures were fed with 1 ml of CM containing IL-2. The lymphoid cells were harvested on days 9 to 10 when they grew to a high density, washed twice and resuspended in HBSS for adoptive immunotherapy or in CM for in vitro cytotoxicity assay, further expansion, and cryopreservation.

Long term expansion of IVS cells. For further expansion of IVS cells, 2×10^5/well of the IVS cells generated in the initial IVS culture were co-cultured with irradiated (20 Gy) MCA 105 tumor cells ($1\sim2\times10^5$/well) in the presence of RIL-2 (40 U/ml). The expanded cells were harvested and cell concentration was adjusted for long-term expansion. The culture was repeated every 6 to 7 days up to 89 days.

Generation of lymphokine-activated killer (LAK) cells. LAK cells were generated from spleen cells of normal B6 mice, as previously described[13)]. Briefly, spleen cells (4×10^6/well) were cultured in CM in the presence of RIL-2 (1,000 U/ml) for 3 to 5 days.

Chromium-51 release assay for cytotoxicity. The 4-hr chromium-release assay was employed to measure in vitro cytotoxicity against target cells[14)]. The assay was performed in triplicate in

96-well, round-bottomed plates (No. 25850 ; Corning). All target cells were labeled with $Na^{51}CrO_4$ (Japan radio-isotope Inst., Tokyo, Japan) for 60 min and were washed 3 times with CM. Each well contained 1×10^4 labeled tumor targets and various numbers of effector cells in a volume of 300 μl of CM. Plates were spun at 80× g for 3 min before incubation at 37℃ for 4 h. After incubation, the plates were centrifuged at 400× g for 5 min, the supernatant (120 μl) collected and radioactivity counted. The percent lysis was calculated as follows:

$$\frac{\text{Experimental cpm}-\text{Spontaneous cpm}}{\text{Maximal cpm}-\text{Spontaneous cpm}}\times100$$

Spontaneous cpm was the amount of ^{51}Cr released from target cells in the absence of effector cells. Maximal cpm was the amount of ^{51}Cr released from the target cells lysed by addition of Triton-×100.

Adoptive immunotherapy model. B6 mice were injected i. v. with 1×10^6 MCA 105 tumor cells in 1.0 ml of HBSS to initiate pulmonary metastases. On day 3 when microscopic foci in the lung became evident, IVS lymphoid cells were given i. v. in HBSS through tail vein. Mice were also injected i. p. with RIL-2 (15,000 U) in 0.5 ml of HBSS, twice a day for 3 days to maximize the anti-tumor effect as previously indicated[7)~9)]. On days 19 to 24 after tumor inoculation, mice were ear-tagged, randomized, then sacrificed for enumeration of pulmonary tumor nodules in double-blind fashion as described elsewhere[15)]. Metastatic foci too numerous to count were assigned an arbitrary value of 250 because this was the greatest number of nodules that could be reliably enumerated. In all experiments, each group consisted of at least five.

Statistical analysis. The significance of differences in numbers of pulmonary metastases between different groups was analyzed by the Wilcoxon rank-sum test[16)]. Two-sided p values were presented in all experiments. No mice were excluded from the statistical evaluations.

Results

Feasibility of cryopreservation of stimulator tumor cells and responder lymphoid cells. The IVS culture was carried out using cryopreserved fresh tumor cells and lymphocytes from tumor-bearing mice. Frozen period of cells used in the present study was between 1 month to 6 months. Recovery of viable cells after thawing was 60 to 95%. Frozen tumor cells and lymphocytes obtained from 11 day-tumor bearing mice were rapidly thawed in 37℃ water bath, then washed with CM. Thawed lymphocytes were co-cultured with tumor cells (20 Gy-irradiated) in the presence of RIL-2. After 9 days of culture, lymphoid cells were harvested and tested in vivo therapeutic efficacy. As shown in table 1, adoptive transfer of the IVS cells generated from frozen tumor stimulator cells and lymphocytes could effectively mediate the regression of 3-day pulmonary metastases. While the mean number of pulmonary metastases in no treatment group and treated with IL-2 alone was 227 and 250 respectively, treatment with IVS cells and IL-2 resulted in the mean number of pulmonary metastases of 16.

Antigenic efficacy of cryopreserved fresh and cultured MCA 105 tumor cells in IVS culture. Cryopreserved fresh and cultured MCA 105 tumor cells were thawed, irradiated and then used as tumor stimulator cells in IVS culture. Two independent experiments were summarized in Table 2. Tumor-drained lymph node cells (EXP. 1 : 13 days, EXP. 2 : 15 days) were co-cultured with tumor

Table 1 Adoptive Immunotherapy of 3 Day Pulmonary Metastases with the IVS Cells Generated from Cryopreserved Responder Lymphocytes and Tumor Stimulator Cells

Group	Cells transferred[a]	No. cells[b]	IL-2[c]	Mean number of pulmonary metastases (SEM)[d]
1	−		−	227 (23)
2	−		+	250
3	TB-IVS	5×10^6	+	16 (12)[e]

[a]Frozen fresh MCA 105 tumor cells and popliteal lymph node cells obtained from mice bearing intra-footpad MCA 105 tumors for 11 days were rapidly thawed in 37℃ water bath, washed, and resuspended in CM. Effector cells (TB-IVS) were generated from thawed lymph node cells (4×10^5/well) and tumor cells (2×10^5/well, 20 Gy-irradiated) co-cultured in the presence of RIL-2 (40 U/m*l*) for 9 days.

[b]B6 mice were injected i. v. with 1×10^6 MCA 105 tumor cells suspended in 1.0 m*l* of HBSS. On day 3, IVS effector cells suspended in 1.0 m*l* of HBSS were given i. v. to each mouse.

[c]RIL-2 (15,000 U) diluted with 0.5 m*l* of HBSS were given i. p. twice a day from day 3 to day 5.

[d]On day 21, mice were ear-tagged, randomized and sacrificed for enumeration of metastatic pulmonary nodules.

[e]Significantly different from groups with no treatment or treated with IL-2 alone ($p<0.05$).

Table 2 Antigenic Efficacy of Cyropreserved Fresh and Cultured MCA 105 Tumor Cells for the Generation of Therapeutic IVS Cells

Group	Tumor stimulator in IVS culture[a]	IL-2[b]	Mean number of pulmonary metastases (SEM)[c]	
			EXP. 1	EXP. 2
1		−	129 (70)	250
2		+	211 (39)	219 (31)
3	Fresh	+	0[d]	8 (2)[d]
4	Cultured	+	<1 (1)[d]	27 (19)[d]

[a]Popliteal lymph node cells (4×10^5/well) obtained from mice bearing MCA 105 tumors for 13 days (EXP. 1) or 15 days (EXP. 2) were co-cultured with either frozen fresh (Fresh) or frozen cultured (Cultured) MCA 105 tumor cells (2×10^5/well, 20 Gy-irradiated) in the presence of RIL-2 (40 U/m*l*) for 9 days. B6 mice were injected i. v. with 1×10^6 MCA 105 tumor cells. On day 3, IVS cells were given i. v. to each mouse.

[b]RIL-2 (15,000 U) diluted in 0.5 m*l* of HBSS were given i. p. twice a day from day 3 to day 5.

[c]On day 19 (EXP. 1) and day 22 (EXP. 2), mice were ear-tagged, randomized, and sacrificed for enumeration of pulmonary metastatic nodules.

[d]Significantly different from groups with no treatment or treated with IL-2 alone ($p<0.05$).

stimulators in the presence of RIL-2 for 9 days. The effector cells generated from IVS culture with cryopreserved either fresh or cultured MCA 105 tumor cells could effectively mediate the regression of 3-day pulmonary metastases in both experiments.

Feasibility of cryopreservation of IVS effector cells. The therapeutic efficacy of cryopreserved IVS cells was evaluated. The effector IVS cells were generated from 13 day tumor-draining popliteal lymph node cells co-cultured with cryopreserved MCA 105 tumor cells in the presence of RIL-2. After 9 days of IVS culture, the lymphoid cells were harvested and cryopreserved. When used, the cryopreserved IVS cells were thawed, washed twice with HBSS and adoptively transferred i. v. into mice with 3-day pulmonary metastases. Normal spleen cells, tumor-draining lymph node cells and spleen cells obtained from 15 day tumor-bearing mice were also co-cultured with MCA 105 tumor stimulator cells in the presence of RIL-2. After 9 days of culture, effector cells generated were harvested and immediately transferred into mice with 3-day pulmonary metastases. As shown in Table 3, adoptive transfer of 3×10^6 IVS cells generated from normal spleen cells had no therapeutic effect, reproducing previous results[7)~9)]. In contrast, IVS cells generated from lymph node cells and spleen cells obtained from tumor-bearing mice were capable of reducing metastatic

Table 3 Therapeutic Efficacy of Cryopreserved IVS Cells

Group	Cells transferred[a]	No. cells[b]	IL-2[c]	Mean number of pulmonary metastases (SEM)[d]	
				EXP. 1	EXP. 2
1	—		−	43 (12)	220 (12)
2	—		+	31 (5)	176 (23)
3	N-spl IVS	3×10^6	+	60 (22)	232 (6)
4	TB-LN IVS	3×10^6	+	2 (1)[e]	0[e]
5	TB-spl IVS	3×10^6	+	0[e]	2 (1)[e]
6	Frozen IVS	3.7×10^6	+	2 (2)[e]	0[e]

[a]Tumor-draining popliteal lymph node cells (TB-LN) and spleen cells (TB-spl) obtained from 15-day MCA 105 tumor-bearing mice were cultured with MCA 105 tumor stimulator cells in the presence of RIL-2 for 9 days. Spleen cells obtained from normal B6 mice (N-spl) were used as control. Frozen IVS cells were generated from lymph node cells obtained from 13-day MCA 105 tumor-bearing mice cultured with MCA 105 tumor cells in the presence of RIL-2. The effector cells were cryopreserved. On the day of adoptive transfer, the cryopreserved IVS cells were rapidly thawed in 37℃ water bath, and immediately transferred i. v. to mice with established 3-day pulmonary metastases.

[b]B6 mice were injected i. v. with 1×10^6 MCA 105 tumor cells suspended in 1.0 m*l* of HBSS. On day 3, IVS cells (3×10^6 and 3.7×10^6) were given i. v. to each mouse.

[c]RIL-2 (15,000 U), diluted in 0.5 m*l* of HBSS was given i. p. twice a day from day 3 to day 5.

[d]On day 24 (EXP. 1) or 18 (EXP. 2), mice were ear-tagged, randomized, and sacrificed for enumeration of metastatic pulmonary nodules.

[e]Significantly different from groups with no treatment, treated with RIL-2 alone or treated with N-spl IVS cells with RIL-2 ($p<0.05$).

nodules from a mean of 43 and 31 (no treatment and IL-2 alone, respectively) in control groups, to less than 2, confirming the results presented in Table 1. Furthermore, adoptive transfer of the IVS cells right after thawing was equally effective to reduce pulmonary metastases down to 2.

Requirement of antigenic stimulation during further expansion of IVS cells. It has been demonstrated that specific antigenic tumor stimulation during the initial IVS culture is essential for the generation of therapeutic effector cells[8]. As the first step for the long-term expansion of therapeutic cells, the requirement of antigenic stimulation during further expansion of IVS cells was evaluated. The IVS culture was initiated with cryopreserved tumor-draining lymph node cells and spleen cells with cryopreserved fresh or cultured MCA 105 tumor cells (20 Gy irradiated) in the presence of RIL-2. After 9 days of culture, lymphoid cells were harvested, washed with CM and then further cultured with or without irradiated MCA 105 tumor cells in the presence of RIL-2. Cells further expanded were harvested 5 days after culture and tested their therapeutic efficacy in vivo using 3-day pulmonary metastases model. Two independent experiments were summarized in Table 4. In both experiments, cryopreserved tumor-draining popliteal lymph node cells and spleen cells obtained from 12-day tumor-bearing mice were thawed and used for IVS culture. Cryopreserved fresh and cultured MCA 105 tumor cells were used in EXP. 1 and EXP. 2, respectively. The results of two experiments clearly demonstrated that while IVS cells further expanded without additional

Table 4 Antigenic Requirement during Further Expansion of Therapeutic Cells

Cells transferred[a]	Tumor stimulator in further expansion[b]	IL-2[c]	No. of pulmonary metastases (SEM)[d]	
			EXP. 1	EXP. 2
−		−	219 (31)	161 (55)
−		+	250	242 (8)
TB-LN IVS	−	+	137 (47)	202 (32)
TB-LN IVS	+	+	0[e]	1 (1)[e]
TB-spl IVS	−	+	N. D.	198 (41)
TB-spl IVS	+	+	N. D.	8 (5)[e]

[a]IVS effector cells were generated from lymph node cells (TB-LN IVS) and spleen cells (TB-spl IVS) obtained from 12-day MCA 105 tumor bearing mice.

[b]IVS cells generated in 9-day initial IVS culture were harvested and further expanded either with RIL-2 alone or with additional MCA 105 tumor stimulator cells (20 Gy-irradiated) in the presence of RIL-2 for 5 days. These expanded IVS cells (3×10^6 ; EXP. 1, 4×10^6 ; EXP. 2) were transferred to mice bearing 3-day pulmonary metastases to evaluate their therapeutic efficacy in vivo.

[c]RIL-2 (15,000 U) diluted in 0.5 m*l* of HBSS was given i. p. twice a day for 3 days after cellular transfer.

[d]Lungs were harvested and number of metastases were counted 19 days (EXP. 1) and 18 days (EXP. 2) after tumor inoculation.

[e]Significantly different from groups with no treatment or treated with RIL-2 alone ($p<0.05$).

tumor stimulation lost their therapeutic efficacy, IVS cells re-stimulated with MCA 105 tumor cells for 5 days in the presence of RIL-2 maintained their anti-tumor effect in vivo, indicating the necessity of periodic antigenic tumor stimulation for expansion of therapeutic IVS cells.

Long term expansion of IVS effector cells. Since periodic antigenic tumor stimulation was essential to expand IVS cells with anti-tumor effect in vivo, we next examined if IVS cells can be expanded, maintaining their therapeutic efficacy for longer period. IVS cells were generated from lymph node cells and spleen cells obtained from 13 day tumor-bearing mice co-cultured with cryopreserved cultured MCA 105 tumor cells in the presence of RIL-2. After 10 days of initial IVS culture, cells were harvested and counted. Two $\times 10^5$/well of IVS cells were cocultured with cryopreserved cultured MCA 105 tumor cells (1×10^5/well, 20 Gy-irradiated) in the presence of RIL-2 for further expansion. This expansion procedure was repeated every 5 to 7 days for about 3 months and the anti-tumor effect of longterm expanded IVS cells in vivo was assessed. The IVS cells generated from lymph node cells obtained from 11 day tumor-bearing mice cultured with tumor stimulator cells for 9 days were used as positive control. As shown in Table 5, adoptive transfer of 4×10^6 longterm (89 days) cultured IVS cells could effectively mediate the regression of pulmonary metastases, but the anti-tumor effect required exogenous IL-2 administration.

In vitro cytotoxic activity of long term expanded IVS cells against fresh tumor targets. The cytotoxicity of the IVS cells generated in the initial 9-day culture, as well as in long-term culture was assessed by standard 4 hr chromium-release assay. Freshly harvested MCA 105 tumor cells were used as autologous tumor targets and MCA 102 tumor cells were served as specific control targets. In vitro cytotoxic activity of effector cells was summarized in Figure 1. The IVS cells generated from MCA 105 tumor-bearing mice in the initial 9-day IVS culture (TB-LN IVS) showed higher cytolytic activity against autologous MCA 105 tumor targets, compared with fresh non-cultured lymph node cells obtained from MCA 105 tumor bearing mice (Fresh TB-LN) (Figure 1.

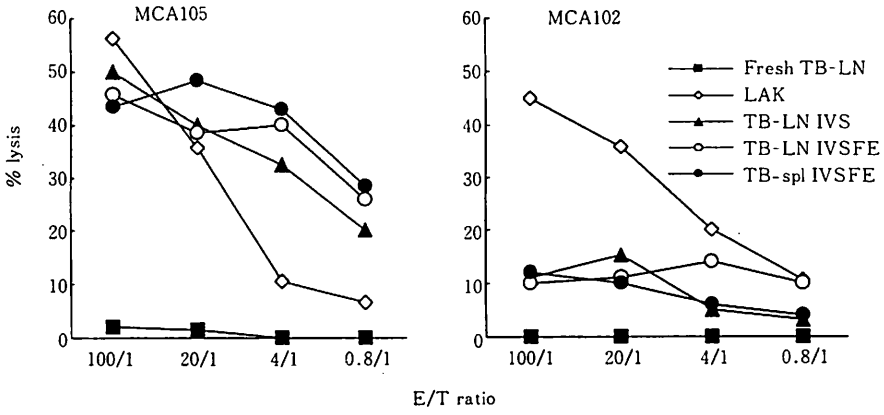

Fig. 1 In Vitro Cytotoxic Activity of IVS Cells
IVS effector cells used in the experiment were identical used in Table 5. Fresh non-cultured popliteal lymph node cells obtained from 13-day MCA 105 tumor bearing mice (fresh TB-LN) and lymphokine-activated killer cells (LAK) were used as control.

left). In contrast, the cytotoxic activity of the IVS cells against MCA 102 tumor cells was marginal (Figure 1. right). The longterm cultured IVS cells generated from either tumor-draining lymph node cells (TB-LN IVS further expanded ; FE) or spleen cells (TB-spl IVS FE) of tumor-bearing mice also showed specific cytotoxic activity against autologous MCA 105 tumor targets.

Discussion

In the present study, we have demonstrated several important points for clinical applications of the IVS culture. First, the lymphocytes obtained from tumor-bearing mice and fresh autologous tumor cells as well as established autologous tumor cell lines could be successfully cryopreserved and used as responder lymphocytes and tumor stimulator cells in the IVS culture. Since recovery of patients' general condition after surgery varies in each case, this fact would be of particularly important for controlling the time to initialize the IVS culture in each case. The feasibility of the IVS cells in effector phase level would also be important in cases when the general condition of the patients was changed and cellular transfer had to be postponed. Furthermore, these facts would enable us to combine the adoptive immunotherapy with chemotherapy and/or irradiation therapy.

In the previous study, we have demonstrated that large number of autologous tumor stimulator cells was required in the IVS culture for the generation of therapeutic effector cells from tumor-bearing mice[8]. In clinical situation, it would be difficult to get enough fresh autologous tumor cells from surgically removed tumor tissues)[17)~19)]. Since the established autologous tumor cell line could

Table 5 Long-term Expansion of IVS Effector Cells

Cells transferred[a]	No. of cells[b]	IL-2[c]	Mean number of pulmonary metastases (SEM)[d]
—		−	250
—		+	250
TB-LN IVS	4×10^6	+	1 (1)[e]
TB-LN IVS FE	4×10^6	+	20 (3)[e]
TB-LN IVS FE	4×10^6	−	172 (45)
TB-spl IVS FE	4×10^6	+	0[e]

[a]IVS effector cells were generated from lymph node cells obtained from 11-day MCA 105 tumor bearing mice (TB-LN IVS). For long-term expansion of IVS cells, IVS cells generated from tumor-draining lymph node cells (TB-LN IVS further expanded ; FE) and spleen cells (TB-spl IVS FE) obtained from 13-day MCA 105 tumor-bearing mice were cultured with periodic antigenic tumor stimulator in the presence of RIL-2 for 89 days.

[b]B6 mice were injected i. v. with 1×10^6 MCA 105 tumor cells. On day 3, IVS cells were given i. v. to each mouse.

[c]RIL-2 (15,000 U) diluted in 0.5 m*l* of HBSS was given i. p. twice a day from day 3 to day 5.

[d]Lungs were harvested and number of metastases were counted 21 days after tumor inoculation.

[e]Significantly different from groups with no treatment or treated with RIL-2 alone ($p<0.05$)

supply virtually unlimited tumor stimulator in IVS culture (Table 2), it would be possible to wider the application of the IVS cell trasfer treatment.

The effector cells generated in the initial 9-day IVS culture could be expanded by periodic antigenic stimulation with autologous tumor cells in the presence of RIL-2, while maintaining their anti-tumor efficacy in vivo for about 3 months (Table 5). As successful adoptive immunotherapy requires large number of syngeneic effector cells[1)~6)], capability of longterm expansion of the IVS cells would give us a fairy good chance to transfer larger number of therapeutic cells over longer time period. In several animal medels, the characterization of longterm cultured effector cells have been reported. Using FBL-3 model, we have deomonstarted that tumor specific cytotoxic clone could be generated from tumor-bearing mice by mixed lymphocyte-tumor culture followed by cloning with limiting-dilution technique. The FBL-3 specific T cell clone was $CD8^+$ and showed in vivo anti-tumor activity and in vitro cytotoxic activity against FBL-3 targets[20)]. The in vivo activity of the T cell clone appeared to be dependent on exogenous IL-2 administration, which was also observed in the present study (Table 5). These data suggested that exogenous IL-2 administration would be essential for enhancing anti-tumor effect in clinical protocol.

Previous studies demonstrated that initial 9-day cultured IVS cells were $CD8^+$ and showed cytotoxic activity against autologous tumor targets, as well as non-specific tumor targets [8),21)]. Long term cultured IVS cells were also $CD8^+$ and showed cytolytic activity against autologous tumor target (Figure 1, data not shown). From these data, tumor regression by the transfer of the initial 9-day IVS cells as well as long-term cultured IVS cells seemed to be mediated, at least in some parts, through direct tumor cell cytolysis.

Recently, we also have developed sensitization procedure where lymphoid cells from tumor-bearing mice were stimulated with anti-CD3 monoclonal antibody followed by culture with RIL 2[22),23)]. This anti-CD3 activated T (αCD3-AT) cells showed specific anti-tumor activity in vivo. Phenotypical analysis of αCD3-AT cells revealed that tumor rejection after cellular transfer was mediated by $CD8^+$ effector cells, but they did not show significant cytolytic activity against autologous tumor targets in vitro[23)~25)]. These data suggested, although tumor regression required the same $CD8^+$ effector T cells, IVS cells and αCD3-AT cells may have different role in tumor destruction in vivo. The histological analysis of the tumor regression mediated by the IVS cells and αCD3-AT cell is now underway in our laboratory.

The MCA 102 fibrosarcoma has been shown to be non-immunogenic in syngeneic host[26)]. We have previously demonstrated that therapeutic IVS cells could be generated by the combination of active immunization with viable MCA 102 tumor cells and bacterial adjuvant (Corynebacterium parvum) followed by the IVS culture[27)]. This active immunization and IVS culture protocol may give us more realistic hope for applying the adoptive immunotherapy protocol in human cancer. Furthermore, our preliminary data have indicated that periodic antigenic tumor stimulation was essential for the long-term expansion of therapeutic αCD3-AT cells (data not shown). In this situation, the data presented in the current study describing the feasibility of cryopreservation of tumor cells would be more significant for applying the adoptive immunotherapy for more cancer patients.

Taken together, our results indicated that the IVS culture, as well as αCD3 activation culture can be applied for more patients with cancer in rather ideal timing and combination with other

treatment modalities to improve therapeutic efficacy.

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In Vitro Sensitization (IVS) 培養法による，担癌宿主からの抗腫瘍エフェクターT細胞の誘導：エフェクター前駆細胞，抗原腫瘍細胞とエフェクターT細胞自身の凍結保存性および，エフェクターT細胞の長期培養に関する研究

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要 旨

同系マウスにおいて弱免疫原性である，メチルコラントレン誘発線維肉腫，MCA 105，106を用い，我々は，担癌宿主由来リンパ球から，in viro sensitization (IVS) 培養法により，in vivo で高い治療効果を発揮するエフェクターT細胞の誘導・増殖が可能であることを報告してきた．今回，IVS 培養法の現実的な臨床応用を前提に，担癌宿主由来リンパ球，抗原腫瘍細胞，さらに IVS 培養によって誘導・増殖されたエフェクターT細胞自身の凍結保存性と，エフェクターT細胞の長期培養の可能性について検討した．担癌7—14日のマウスから得られた腫瘍所属リンパ節細胞および脾臓細胞は，凍結保存後も IVS 培養によって in vivo での抗腫瘍効果を発揮するエフェクター細胞に誘導可能であった．新鮮腫瘍細胞，および in vitro で樹立された腫瘍細胞株も免疫原性を保持したまま，凍結保存可能であった．さらに，IVS エフェクター細胞自身も in vivo での治療効果を保持したまま，凍結保存可能であった．IVS エフェクター細胞の長期培養では，in vivo での抗腫瘍効果維持のためには周期的腫瘍抗原刺激および RIL-2 の添加が必要であったが，腫瘍抗原刺激と RIL-2 により，約3カ月間，治療効果を保持した状態で培養可能であった．In vitro では長期培養後も自己腫瘍細胞に有意な腫瘍細胞傷害活性を示した．以上の結果より、IVS エフェクター細胞培養開始時期の選択が可能となり，他の治療法との併用や，長期培養による，大量のエフェクター細胞の移入が可能となり，効果的な養子免疫療法の可能性が拡大された．

索引用語：養子免疫療法，In Vitro Sensitization (IVS)，凍結保存，エフェクター細胞長期培養，肺転移