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Solid-phase Anti-CD3 Antibody Activation and Cryopreservation of Human Tumor-infiltrating Lymphocytes Derived from Epithelial Ovarian Cancer

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The effect of solid-phase anti-CD3 antibody activation and cryopreservation was evaluated on thirteen samples of tumor-infiltrating lymphocytes (TILs) derived from epithelial ovarian cancer. Seven preparations of TILs were cultured with or without solid-phase anti-CD3 antibody in addition to 100 units/ml of recombinant interleukin-2 (rIL-2). The proliferation rate of all of the seven TIL preparations stimulated by anti-CD3 antibody on the fourth or fifth day of culture was 3.4 to 9.8 times greater than that of lymphocytes cultured with rIL-2 alone. Furthermore, in an experiment with five TIL samples activated with anti-CD3 antibody, three of them showed augmented cytotoxic activity against autologous fresh tumor cells. The population of $CD3^+/CD8^+$ TILs was increased after 4–5 weeks of cultivation and $CD8^+$ lymphocytes amounted to over 70% in all of seven preparations tested, whereas two of seven preparations not activated by anti-CD3 antibody were $CD3^+/CD4^+$-dominant. In addition, nine preparations of TILs cultured with rIL-2 were cryopreserved for several weeks; after recovery from cryopreservation, no major change was observed in cell surface markers, in growth rate or in cytotoxic activity. These results suggest that cryopreserved and/or anti-CD3 antibody-activated lymphocytes could conveniently be employed in a clinical trial of adoptive immunotherapy employing TIL.

Key words: Tumor-infiltrating lymphocyte — Anti-CD3 antibody — Cryopreservation

Although chemotherapy with a cisplatin-containing regimen is reported to be relatively effective in the treatment of epithelial ovarian cancer, the median survival rate for patients at an advanced stage is only about two years.[1,2] This fact indicates that a novel anti-cancer therapy is needed as an adjunct to present treatments, i.e., operation, chemotherapy and radiotherapy, to eliminate the small numbers of residual cancer cells which become refractory to the conventional treatments. Following reports that the administration of tumor-infiltrating lymphocytes (TILs) and recombinant interleukin-2 (rIL-2) produced higher response rates than those achieved by administration of rIL-2 alone or administration of rIL-2 with LAK cells in the treatment for metastatic melanoma,[3,4] several clinical trials have been performed.[5,6]

In our previous clinical trials of adoptive immunotherapy employing autologous TILs to epithelial ovarian cancer, of seven patients with advanced or recurrent disease, one had a complete response and four had a partial response.[7] However, the duration of response was only 3–6 months and four patients died of tumor regrowth. This result indicates that this immunotherapy may produce more favorable clinical outcomes when used in combination with chemotherapy or radiotherapy. To maintain the anti-cancer effects of chemotherapy, and further to improve the prognosis of the patients, we have to establish a suitable protocol for combination therapy of anti-cancer drugs and TIL.

In this study, in an attempt to harvest a large enough number of TILs to kill all tumor cells, we first examined the effects of solid-phase anti-CD3 antibody stimulation on TIL, since solid-phase anti-CD3 antibody has recently been reported to activate and proliferate murine and human TILs.[8,9] Secondly, we examined the influence of cryopreservation to see if cryopreserved TILs can be used in the clinical setting, e.g. in prevention of the immunosuppressive state after chemotherapy is finished.

MATERIALS AND METHODS

Culture of TIL Thirteen TIL preparations derived from epithelial ovarian cancer were cultured, and seven and nine of them were used for the studies of anti-CD3 antibody activation and cryopreservation, respectively. Discarded human tumor tissue was obtained from the Department of Obstetrics and Gynecology, Niigata University Hospital. Each tumor was divided into small pieces with scissors and was further dissociated with an enzyme solution composed of 0.01% hyaluronidase type V (1500 U/g), 0.1% collagenase type IV (163 to 230 U/g), and 0.002% deoxyribonuclease type I (100 U/g) (Sigma Chemical Co., St. Louis, MO). The cells were washed twice with Hanks' balanced salt solution (HBSS) without Ca^{2+} and Mg^{2+}, and cellular debris was removed from bulk cultures by centrifugation on a Ficoll lymphocyte separation medium (Litton Bionetics, Charleston, SC) density gradient. Viable cells were then counted and

placed in complete culture medium in 6-well tissue culture plates (Costar). The standard culture consisted of viable cells in 6-well plates at 2×10^5 cells/ml in complete culture medium under a humidified atmosphere containing 5% CO_2 at 37°C. The complete culture medium used was RPMI 1640, containing 2% human AB serum, 2 m*M* glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, 50 μg/ml gentamicin (M.A. Bioproducts, Walkersville, MD), and 100 U/ml of rIL-2 (Shionogi & Co., Ltd., Osaka).

Solid-phase activation Monoclonal anti-CD3 antibody (generously provided by Jansen Kyowa Co., Ltd., Tokyo) was diluted to the concentration of 5 μg/ml with sterile 0.2 *M* carbonate buffer (pH 8.6). Diluted antibody was dispensed over the surfaces of the wells of 6-well tissue culture plates and the plates were stored horizontally at 4°C. Prior to use, the antibody was removed and the solid-phase was washed three times with HBSS.[8] For activation, single cell suspensions of tumor cells and lymphocytes were resuspended in RPMI-1640 without exogenous IL-2, and dispensed onto the coated surfaces at 1×10^6 cells/5 ml/well and the plates were incubated at 37°C. After 4 days, the cells were removed, washed twice with HBSS, plated into 6-well plates at the density of 2×10^5 cells/ml in complete medium, and treated with rIL-2 at the concentration of 100 U/ml. Every 4 to 7 days, cultures were split by cell redistribution into fresh plates with addition of new medium.

Cryopreservation of TIL After 4 to 5 weeks of cultivation with 100 U/ml of rIL-2 alone, nine preparations of TILs were frozen in 30% human AB serum with 7.5% dimethylsulfoxide, using a Programing Freezer (Planer Products Ltd.) and cryopreserved in liquid nitrogen for several weeks. Then they were thawed rapidly at 37°C, washed twice with HBSS without Ca^{2+} and Mg^{2+}, and placed in 6-well tissue culture plates (Costar) in complete culture medium, as described above.

Flow cytometry All TILs (1×10^5 cells) were washed in HBSS free from phenol red, containing 1% fetal calf serum and 0.2% sodium azide at 4°C and then were stained with the appropriate fluorescein isothiocyanate (FITC)-labeled monoclonal antibody, incubated at 4°C for 45 min, washed twice, and resuspended in 0.5 ml of medium for FACS analysis. The monoclonal antibodies used were: CD3, CD4, CD8, CD16, CD25, and HLA-DR (Becton Dickinson Japan, Tokyo).

***In vitro* cytotoxicity** The *in vitro* cytotoxicity of TIL was assessed by using fresh-frozen autologous and allogeneic tumor cells as targets. The targets were labeled with carboxyfluorescein diacetate (C-FDA; Sigma Chemical Co.) (50 μg/ml diluted with RPMI 1640) for 60 min at 37°C and washed three times with HBSS. Then the target cells (5×10^3 cells/well) were incubated with various numbers of effector cells at 37°C for 3 h. C-FDA in the supernatant was masked with 5 m*M* calf hemoglobin (Wako Pharmaceuticals Ltd., Tokyo) and C-FDA in surviving cells was counted on an automated fluorescence microscope photometer (Reitz compact-MT). Results were converted to percent specific cytotoxicity: % specific toxicity = (maximal C-FDA count − test C-FDA count) / (maximal C-FDA count − minimal C-FDA count). Target cells incubated in medium alone or 2% Triton X-100 were used to determine spontaneous and maximal C-FDA count, respectively. Conversion to lytic units was performed as described previously.[9] One LU_{50} was defined as the number of effector cells mediating 50% specific lysis of 5×10^3 target cells.

Statistical analysis Differences in mean expansion index were analyzed using Student's *t* test. Differences in cell phenotypes and percentages of specific cytotoxicity were determined by the Wilcoxon rank sum test.[10]

RESULTS

Anti-CD3 antibody activation Seven TIL preparations under standard culture conditions were activated with anti-CD3 antibody and their proliferation was studied. From each TIL culture, a representative sample was collected 2, 4 and 6 weeks after cultivation. The samples were purified using Ficoll, and counted using the trypan blue exclusion method to determine whether the proliferation rate of TIL could be modified by anti-CD3 antibody activation.

At the point of maximal culture proliferation, all seven cultures activated with anti-CD3 antibody showed cell yields approximately 3.4 to 9.8 times greater than those obtained under standard conditions. Figure 1 shows the comparative growth curves for both culture conditions for seven TIL preparations from epithelial ovarian cancers, indicating that anti-CD3 antibody activation significantly improved the proliferating capability of TIL. This effect was apparent 2 weeks after the start of cultivation and lasted for 4 weeks.

To observe the changes in the cellular surface phenotypes of TILs, flow cytometry was performed on standard cultured and anti-CD3 antibody-activated TILs after 2 to 6 weeks of cultivation. $CD3^+$ T lymphocytes were clearly recognized as the major cell type in all TIL preparations, and many of them were activated T lymphocytes, as judged by the expression of the HLA-DR antigen. Under standard culture conditions, two of the seven preparations were $CD3^+/CD4^+$ dominant (patients 125 and 130); however, all of seven preparations activated by anti-CD3 antibody were $CD3^+/CD8^+$-dominant (Table I). The population of $CD3^+/CD8^+$ TIL was increased during the process of cultivation, and $CD8^+$ lymphocytes accounted for over 70% after 6 weeks of cultivation in all cases (Table I).

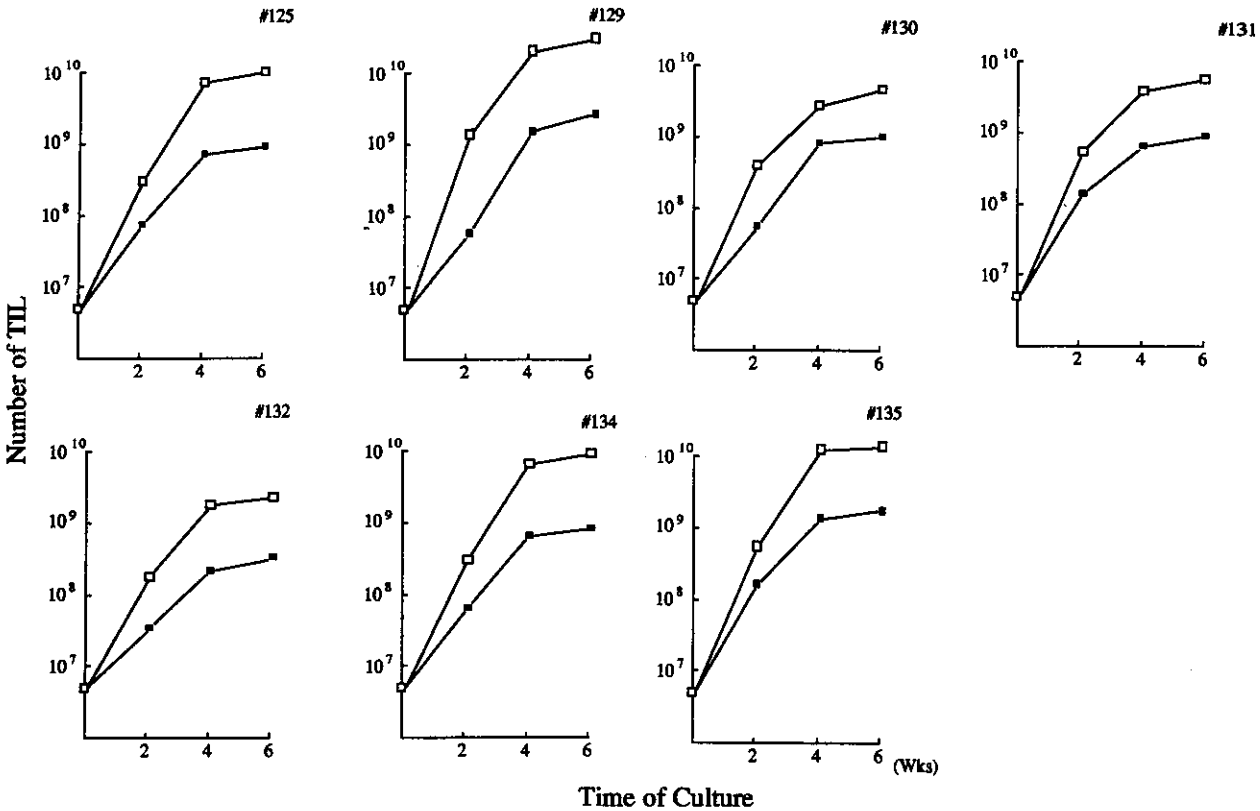

Fig. 1. Comparison of growth curves of standard conditioned and anti-CD3 antibody-activated TILs. Samples show an increased proliferation rate of activated lymphocytes in all cases. Cultures were terminated after 6 weeks. ■: standard conditioned TILs, □: anti-CD3 antibody-activated TILs.

To compare the cytotoxic properties of TILs cultured under standard conditions with those of TILs activated by anti-CD3 antibody, five preparations were tested in a three hour C-FDA cytotoxicity assay at 4 to 5 weeks after initial culture. Under the standard conditions, four preparations, which were $CD3^+/CD8^+$-dominant, showed high cytotoxic activity against autologous fresh tumor cells (20 to 48 $LU_{50}/10^7$ effectors), but not allogeneic tumor cells (7 to 12 $LU_{50}/10^7$ effectors), and one preparation (patient 130), in which over 78.8% of cells were $CD3^+/CD4^+$ TILs, expressed only weak cytotoxicity against either autologous or allogeneic tumor cells. In the anti-CD3 antibody-activated preparations, the cytotoxic activity of three TIL preparations (patients 130, 131 and 132) was enhanced only against autologous tumor cells, while two preparations (patients 129 and 134) showed almost the same cytotoxic activity against autologous and allogeneic fresh tumor cells as that of TILs cultured with rIL-2 alone (Fig. 2). Interestingly, substantial killing activity was induced in TILs of patient 130 by anti-CD3 antibody treatment accompanied with phenotypic change of the cell surface antigens.

Influence of cryopreservation After 4 to 5 weeks of cultivation with 100 U/ml of rIL-2 only, TILs were cryopreserved at −190°C in liquid nitrogen for several weeks and changes in proliferating rate, cell surface phenotype, and cytotoxic activity of these frozen-thawed TILs were evaluated before and after cryopreservation.

The recovery rate from cryopreservation varied (47.5 to 92.0%) regardless of the cell surface phenotype, proliferation rate and cytotoxic activity against fresh tumor cells. A comparison of the expansion index for 14-day cultivation of TIL preparations without cryopreservation and that of frozen-thawed TIL preparations revealed no difference, indicating that after recovery from cryopreservation, TIL retained the same proliferation properties (data not shown). Evaluation of changes in the cellular surface phenotypes of nine frozen-thawed TIL samples was performed by flow cytometric analysis employing appropriate monoclonal antibodies. As shown in Table II, no major change was observed in the cell surface markers, and this demonstrates that the same population of lymphocytes as before was recovered homogeneously, even though the recovery rate in some cases (patients 119

Table I. Phenotypic Flow Cytometric Analysis on TILs with or without Anti-CD3 Antibody-activated Culture

Patient No.[a]	Weeks of culture	% of positive cells					
		CD3	CD4	CD8	HLA-DR	CD16	CD25
125-S	2	98.4	81.4	24.7	96.3	NT[b]	NT
	4	96.3	74.8	30.6	89.3	NT	NT
	6	95.9	73.3	22.0	86.1	NT	NT
125-A	2	100.0	85.1	28.4	84.8	NT	NT
	4	100.0	42.5	58.0	88.8	0.8	8.5
	6	99.8	23.9	77.7	94.1	NT	NT
129-S	4	97.7	28.6	69.3	82.5	1.1	18.1
	8	99.0	19.6	76.3	65.8	2.3	27.0
129-A	2	99.2	68.2	33.3	76.1	0.5	47.1
	4	99.7	34.0	59.6	93.4	1.3	3.0
	6	95.1	16.0	73.8	76.7	4.7	1.3
	8	99.0	14.2	84.6	83.0	1.6	0.4
130-S	4	81.7	78.7	6.5	91.3	NT	NT
130-A	4	97.8	9.8	94.1	98.0	NT	NT
131-S	2	95.7	52.8	40.8	97.4	1.8	NT
	4	99.8	36.1	68.3	98.7	1.4	NT
	6	98.3	37.4	70.1	97.4	3.7	11.4
131-A	2	98.6	49.9	46.1	99.3	1.3	NT
	4	99.2	31.8	69.3	99.2	0.5	NT
	6	99.9	30.9	68.7	83.7	7.1	12.2
132-S	4	77.2	36.3	63.1	84.8	0.4	26.7
132-A	4	99.9	20.8	80.8	91.7	NT	NT
134-S	4	97.9	26.1	67.7	82.5	1.1	18.1
134-A	4	95.1	16.6	73.8	76.7	4.7	1.3
135-S	2	98.7	68.6	30.1	99.4	1.9	29.6
	4	98.2	29.7	38.5	98.9	1.1	4.6
	6	87.3	16.3	46.1	80.9	0.8	7.1
135-A	2	99.4	66.6	38.0	99.3	0.6	22.4
	4	99.7	49.5	26.4	95.8	0.4	0.9
	6	98.1	30.5	71.0	85.4	1.3	0.8

Phenotype of TILs was determined by FACS analysis performed with a 488-nm argon laser on a Becton-Dickinson FACS. FITC-labeled monoclonal antibodies were purchased from Becton-Dickinson Japan and used at the appropriate dilution. Leu-4 recognizes mature T cells (CD3); Leu-3 recognizes class I restrictive cytotoxic/suppressor T cells (CD8); Leu-2 recognizes class II restrictive helper/suppressor inducer T cells (CD4); Leu-11a recognizes NK cells and neutrophils (CD16); IL-2 receptor recognizes T cells activated by PHA, Con A or Leu-4, NK cells, T cells in MCL and HTLV-infected T cell leukemia cells (CD25). HLA-DR recognizes B cells, monocytes, macrophages and activated T cells.
a) S: TILs without anti-CD3 antibody activation, A: TILs with anti-CD3 activation.
b) Not tested.

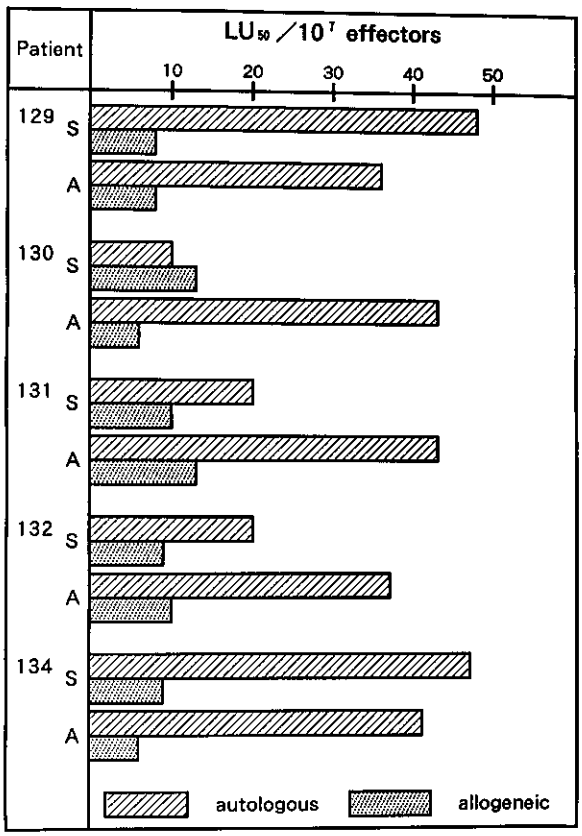

Fig. 2. Results of three-hour C-FDA cytotoxicity assays of standard condition (S) and anti-CD3 antibody activation (A). Among the activated TILs, three (patients 130, 131 and 132) demonstrate greater *in vitro* lytic ability against only autologous tumor cells and no difference was noted in two (patients 129 and 134). One LU is defined as the number of effector cells mediating 50% specific lysis of 5×10^3 target cells.

and 124) was less than 50% after the freeze and thaw cycle.

To investigate the killing activity of frozen-thawed TILs, nine preparations were subjected to three-hour C-FDA cytotoxicity assay on autologous tumor cells. Slightly decreased cytotoxic activity (not statistically significant) was observed in all preparations tested (Fig. 3). Recurrent or metastatic tumor cells in lymph nodes and omentum (patient 125) and in lymph nodes and liver (patient 129) were killed as efficiently as the original tumor cells by frozen-thawed TIL when utilized as target cells of three-hour C-FDA cytotoxicity assay (Fig. 4).

DISCUSSION

The finding that the infiltration of lymphocytes into cancer tissues is a favorable prognostic factor in human cancers has led to trials to expand these lymphocytes and to infuse them into cancer patients.[11] As the culture of TIL for therapeutic application requires the ability reproducibly to obtain tremendously large numbers of lym-

Table II. Comparison of Phenotypic Flow Cytometric Analysis on TILs with or without Cryopreservation

Patient No.	Cryopreservation	% of positive cells					
		CD3	CD4	CD8	HLA-DR	CD16	CD25
118	−	99.6	27.5	76.2	88.2	NT[a)]	NT
	+	98.8	17.3	74.0	92.0	NT	NT
119	−	95.8	23.9	77.7	94.1	NT	NT
	+	94.7	14.9	80.0	80.7	NT	NT
121	−	99.8	27.3	97.0	66.4	1.6	4.7
	+	99.6	30.6	92.5	66.5	1.7	2.7
123	−	95.1	16.6	73.8	76.7	4.7	1.3
	+	98.3	12.4	92.5	89.9	1.8	1.1
124	−	99.9	7.2	89.2	88.2	0.7	1.2
	+	99.5	10.4	89.4	70.8	2.0	0.7
126	−	94.9	52.8	43.5	90.0	NT	NT
	+	96.4	41.4	54.2	87.2	NT	NT
129	−	100	21.6	76.9	97.7	0.6	2.4
	+	100	33.8	79.7	85.5	2.2	2.8
134	−	97.8	19.8	74.1	98.0	NT	NT
	+	99.6	14.8	77.6	95.4	NT	NT
135	−	100	42.5	58.0	88.0	0.8	8.5
	+	99.6	57.1	46.7	92.2	0.0	7.5

Phenotype of TIL was determined by FACS analysis performed with a 488-nm arrgon laser on a Becton-Dickinson FACS. FITC-labeled monoclonal antibodies were purchased from Becton-Dickinson Japan and used at the appropriate dilution. Leu-4 recognizes mature T cells (CD3); Leu-3 recognizes class I restrictive cytotoxic/suppressor T cells (CD8); Leu-2 recognizes class II restrictive helper/suppressor inducer T cells (CD4); HLA-DR recognizes B cells, monocytes, macrophages and activated T cells.
a) Not tested.

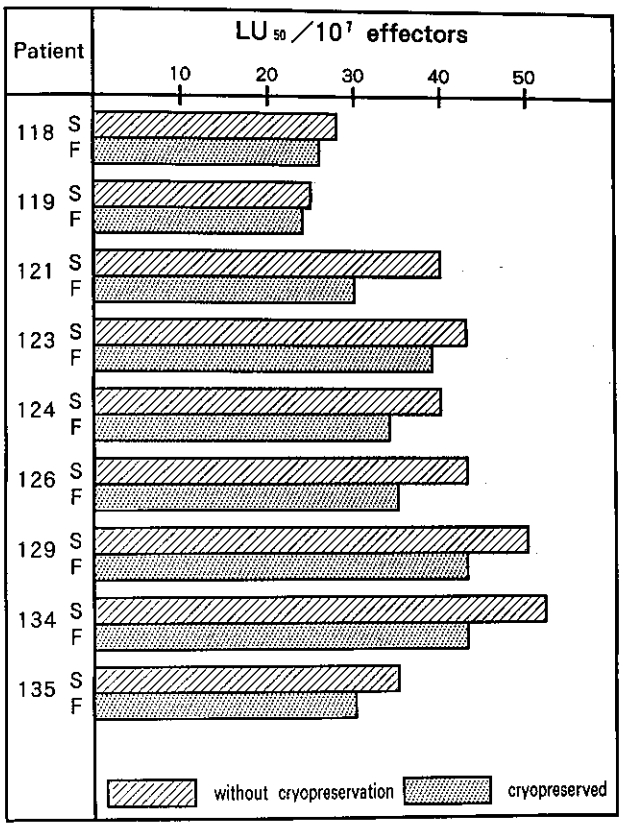

Fig. 3. Three-hour C-FDA cytotoxicity assays of standard condition (S) and frozen-thawed (F) TIL revealed slightly decreased *in vitro* lytic ability (not significant) against autologous tumor cells. One LU is defined as the number of effector cells mediating 50% specific lysis of 5×10^3 target cells.

phocytes (more than 1×10^{10} cells) for adoptive transfer, several protocols[12–14] have been examined based on alternative strategies.

Previous work has demonstrated the ability of anti-CD3 antibodies to activate normal peripheral blood lymphocytes or T cell clones to proliferate, secrete cytokines, become cytotoxic, and express IL-2 receptors.[9, 15–18] These studies have indicated that feeder cells are required for optimal cell activation. However, Deric *et al.* reported an alternative method to activate human TIL by using solid-phase anti-CD3 antibody without the addition of feeder cells.[8]

In the present report, TILs activated with anti-CD3 antibody showed cell yields approximately 3.4 to 9.8 times greater than those of TILs cultured with rIL-2 alone. In addition, we evaluated the cytotoxic activity towards fresh human cancer cells, and found that three of five TIL preparations tested showed augmented cytotoxicity towards autologous tumor cells. These results indicate that a therapeutically useful cell count of TILs can be obtained from a small tumor mass, and thus solid-phase anti-CD3 antibody activation of TILs could be a useful method of *in vitro* TIL cultivation.

Previously, we have shown that the MHC-restricted cytotoxic activity against autologous fresh tumor cells was mediated by $CD3^+/CD8^+$ TILs cultured with a low dosage of rIL-2,[19] in agreement with reports on TIL from melanoma. In this study, all TIL preparations activated by anti-CD3 antibody were $CD3^+/CD8^+$-dominant. However, it is difficult to compare our results with those of previous studies,[20–22] because of the heterogeneity of the cultured TIL infused, and also variations in activating and culture methods. The heterogeneity of cells and the absence of specific cytolytic activity for autologous tumor cells may be a major contributing factor in abrogating or diluting the activity of particular portions of cultured TIL.

From this standpoint, the anti-CD3 antibody activation appears to be a useful approach because of the selective augmentation of the proliferation rate of

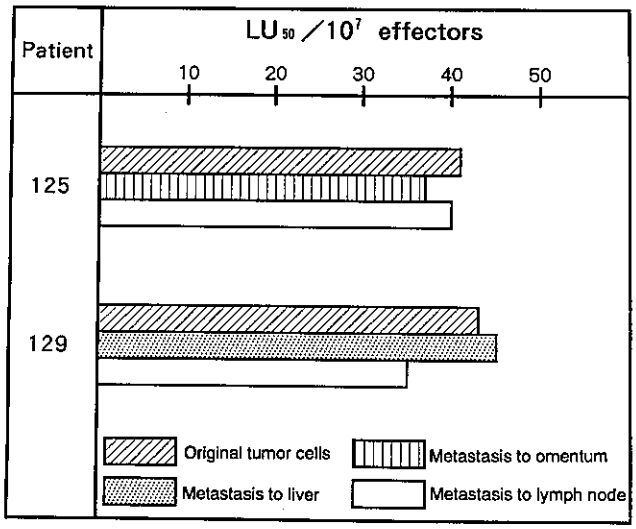

Fig. 4. Changes of cytotoxic activity of frozen-thawed TIL against original and recurrent tumor cells. Tumor cells obtained from metastases to the liver, omentum and lymph node in addition to original tumor cells were employed as target cells for three-hour C-FDA cytotoxicity assay.

$CD3^+/CD8^+$ TILs, though no clinical trial employing anti-CD3 antibody-activated TIL has yet been conducted.

The critical point of adoptive immunotherapy in combination with conventional anti-cancer drugs is to establish adequate conditions, especially as regards timing for infusion of lymphocytes and the injection of anti-cancer drugs (which often possess immunosuppressive effects), because infused lymphocytes have been reported to remain in the tumor sites of patients for several months.[23] In addition, we recently observed enhanced cellular immunity in patients treated with infusion of TIL, as demonstrated by delayed-type hypersensitivity to purified protein derivative (PPD) and to phytohemagglutinin (PHA), and also by NK cytolytic activity towards K562 cells (data not shown). This raises the possibility that observed tumor regression was mediated in part by the activated cellular immunity.

Adoptive immunotherapy using TIL seems to be a promising anti-cancer therapy for killing small numbers of residual tumor cells, if conducted in an appropriate fashion. Since our clinical trials of combined therapy with TILs and cisplatin-containing chemotherapy for patients with advanced and recurrent epithelial ovarian cancer showed an increasing cure rate and long-term survival,[7] we are planning to treat these patients with adoptive transfer of cryopreserved and/or anti-CD3 antibody-activated TILs after all chemotherapy has been completed.

With regard to the influence of cryopreservation on TILs, no major change was noted in growth rate or cell surface markers; slightly decreased, but still valuable cytotoxic activity against autologous fresh tumor cells and even recurrent or metastatic tumor cells was recognized after recovery from cryopreservation. Further investigations into the effect of timing and duration of cryopreservation will enable us to make more efficient use of cryopreserved TILs in the clinical setting.

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