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High Rate of Induction of Human Autologous Cytotoxic T Lymphocytes against Renal Carcinoma Cells Cultured with an Interleukin Cocktail

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A high rate of induction (9 of 10 cases) of human autologous cytotoxic T lymphocytes (CTL) was achieved *in vitro* from peripheral blood mononuclear cells of renal carcinoma patients by applying an interleukin (IL)-cocktail consisting of IL-1, -2, -4, and -6. The CTL specifically lysed their own target carcinoma cells within 24 h but did not kill neighboring autologous normal kidney cells or allogeneic renal cancer cell lines. In the case of TUHR4TKB, for which autologous CTL were not induced, no expression of MHC class-I molecules was observed on the surface of these carcinoma cells, although they were sensitive to autologous natural killer cells. The results imply that adoptive immunotherapy for metastasized renal carcinoma will be feasible with autologous CTL in combination with natural killer cells.

Key words: Autologous CTL — Interleukin — Renal cancer — Immunotherapy

CTL have been suggested as effective killer cells in adoptive immunotherapy for tumors.[1–3] Due to the need for tumor cells or tumor-derived antigens to be present as a continuous stimulation for induction and proliferation of CTL, recent studies have focused mainly on identification of tumor antigens[1–3] and preliminary therapy for tumors.[4, 5] This is presumably because a system for reproducible generation of human autologous CTL has not been established yet, as neither established autologous tumor cell lines nor tumor-derived antigenic peptides for repeated stimulation of the lymphocytes are available. To replace autologous tumor cells, autologous tumor-specific CTL generated using HLA-matched tumor cells[6, 7] or peptide-pulsed antigen-presenting cells[8–10] have been developed. Although there have been many reports of mutations frequently occurring in some specific genes, including genes encoding tumor-related proteins such as p53,[11] the MAGE family,[12] and HER-2/neu,[13] tumor cells show a diversity of mutations in their DNA which may give rise to a variety of antigens.[14–17] To overcome this problem, gene manipulation or hybridization of the tumor cells and antigen-presenting cells has been employed to enhance the antigenicity or to strengthen the interaction between tumor cells and lymphocytes.[17–19] However, these manipulations always require a rather large number of tumor cells and such large amounts are impossible to obtain in many cases of human tumors.[20]

For clinical application of CTL, it is necessary to confirm that human autologous CTL are inducible in most cases. Recently, we established an induction technique involving stimulation with tumor cells and/or fixed tumor sections for alloreactive[21, 22] and autologous[23] CTL by applying a cocktail consisting of 4 ILs.[24] Here, we report that highly reproducible induction of autologous CTL was achieved in renal carcinoma patients, in a study aimed to establish a routine protocol for induction of CTL for renal cancer immunotherapy.

MATERIALS AND METHODS

Reagents and cell lines Recombinant human IL-1b, -2, -4, and -6 were kindly provided by Ohtsuka Pharmaceutical Co., Ltd. (Tokushima), Shionogi & Co., Ltd. (Osaka), Ono Pharmaceutical Co., Ltd. (Osaka), and Ajinomoto, Inc. (Kawasaki), respectively. RHAMα medium was used for culture of human lymphocytes.[25] The enzyme solution used for dissociation of tumor tissues consisted of collagenase type V (500 mg/liter), hyaluronidase type V (50 mg/liter), DNAse type I (12.5 mg/liter), penicillin (2×10^5 IU/liter)), streptomycin (100 mg/liter), and 10%(v/v) FBS in RPMI1640 medium. Monoclonal antibodies for HLA-typing were purchased from One Lamda (Canoga Park, CA), except those against Bw4/A24/A32 (HLA-01 clone, Sanbio Inc., The Netherlands). For detection of MHC-

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Abbreviations: CTL, cytotoxic T lymphocytes; E/T, effector/target; FBS, fetal bovine serum; HLA, human leukocyte antigen; Ig, immunoglolulin; IL, interleukin; LAK, lymphokine-activated killer cells; MHC, major histocompatibility complex; NK, natural killer cells; PBMC, peripheral blood mononuclear cells; PCR, polymerase chain reaction; PE, phycoerythrin; TIL, tumor-infiltrating lymphocytes.

class I, the monoclonal antibody W32/64 was used within 1 week of the primary culture of the tumor cells. The NK-sensitive cell line K562, the LAK-sensitive cell line Daudi, and the renal cancer cell lines OS-RC-2 and Hpt.10 were provided by RIKEN Cell Bank. A gastric carcinoma cell line, GT3TKB, used for the preliminary experiment, was also obtained from routine cultures in RIKEN Cell Bank.

Target renal carcinoma cells Surgically resected specimens of renal carcinomas and associated normal renal tissues removed from a location as far as possible from the carcinoma were minced and dissociated with the enzyme solution at room temperature for 1 h. The mixture was placed on a layered gradient of Ficoll-Paque solutions (100% and 75% Ficoll layers) and centrifuged. Cells at the interface of the 100% and 75% Ficoll layers were collected and cultured in DMEM with 10% FBS. Normal cells and carcinoma cells were subcultured for one passage, and preserved frozen in liquid nitrogen until use in induction of CTL and subsequent experiments. Total cell numbers obtained depended on the tumor tissue size and cell viability, which was influenced by the latent time of the operation and transportation. Usually, 10^6 to 10^7 cells could be obtained for normal cells and tumor cells from resected specimens. However, most of the cells died within the next several days in the primary culture. Some of the carcinoma cells were maintained in DMEM with 10% FBS for several months, with the aim of establishing carcinoma cell lines.

Lymphocyte culture For LAK and NK cultures, PBMC were collected by the conventional method of Ficoll-Paque gradient centrifugation. LAK were prepared by culturing PBMC (1×10^6 cells/ml) in RHAMα medium supplemented with 10% heat-inactivated FBS and 500 U/ml IL-2 for 2 weeks. NK were prepared by culturing PBMC (1×10^6 cells/ml) in the presence of X-ray-irradiated Daudi cells (1×10^5 cells/ml) and IL-2 (50 U/ml) for 2 weeks, as described elsewhere.[26)]

Before addition of the PBMC for CTL induction, confluent renal carcinoma cells maintained in a 6-well plate were irradiated with 30 Gy of X-rays. The PBMC (10^6 cells/ml) were then cultured on these autologous carcinoma cells in the induction medium, i.e., RHAMα medium supplemented just before use with 5% autologous plasma and the IL-cocktail (IL-1b (167 U/ml), IL-2 (67 U/ml), IL-4 (67 U/ml), and IL-6 (134 U/ml)). The CTL induction culture was continued with appropriate changes of the medium (at least half of the medium was changed every 2 days) until the target cells disappeared completely. The CTL were then routinely subcultured on unirradiated autologous target cells at an E/T ratio of 10 in RHAMα medium supplemented with 5% autologous plasma, or 5% heat-inactivated FBS, and the IL-cocktail. The target cells were renewed once a week.

Cytotoxic activity of CTL, LAK, and NK was determined as described[22)] by co-culturing lymphocytes and the target tumor cells for 24 h, except in the cases indicated. The target cells adhering to the bottom of the wells were measured as surviving cells by crystal violet staining. If the lymphocytes were strongly cytotoxic, this assay is as sensitive for assessment of the killing activity as the standard ^{51}Cr-release cytotoxicity assay when it is applied to the adherent target cells (see Fig. 1 of ref. 22). To exclude the possibility of simple growth inhibition of the tumor cells by the CTL, we separately measured the A_{570} of each well of control target cells at the start of the killing assay and this value was taken as 100% in calculation of the percentage of surviving target cells. Note that the tumor cells at the E/T of 0 grew during the 24-h incubation period and therefore showed values higher than 100%. Each point shown is the mean of triplicate observations±SD.

Flow cytometry and HLA-typing CTL and tumor cells were stained with FITC- or PE-labelled antibodies (against human CD3, CD4, CD8, CD56, and MHC class-I) according to the manufacturer's protocol. The stained cells were analyzed by FACScan with LYSIS II software (Becton Dickinson Japan, Tokyo). Serological HLA-typing was performed by flow cytometry after staining with mouse monoclonal antibodies against HLA-A2, Bw4/A24/A32, A1/A11/A26, A11/A24/B41/Cw1, A25/A26, Bw4/A9/A32, A9/A32/A25, A23/A24, and A33/B8, and FITC-labelled goat secondary antibody against mouse IgG or IgM. HLA-subtypes, if required, were determined by PCR with HLA-A2402 allele-specific primer sets.[27)]

RESULTS

Renal carcinoma cells In the present study, we could obtain limited numbers of renal carcinoma cells and we observed growth of corresponding autologous lymphocytes, as shown in Table I. The carcinoma cell lines from patient series Hpt. and Pt. were established in the development stage of the primary culture technique, and only 7 tumor cell lines grew from 38 patients. After the protocol for the routine primary culture (see "Materials and Methods") was set during 1995–1996, 10 cases were submitted to primary renal carcinoma cell culture, and 3 cell lines were established (patient TUHR series in Table I). The expression of MHC-class I was confirmed in all cases shown in Table I, except TUHR4TKB, by conventional flow cytometric analysis. The positively stained carcinoma cells showed an average of 10- to 100-fold higher fluorescence intensity than those stained with FITC-labelled control antibodies (data not shown).

We also obtained the counterpart normal kidney cells from 5 cases in the former series of patients, but from only one case, TUHR10TKB, in the latter series. There-

fore, unfortunately, availability of the autologous full set (i.e., sufficient number of renal carcinoma cells, normal kidney cells, and PBMC from the same patient for induction of CTL and subsequent killing assays) was rare. We could examine only 10 cases, as shown in Table I, including 4 incomplete sets which lack normal kidney cells because of their short life span (i.e., Hpt.10, Hpt.16, TUHR3TKB, and TUHR4TKB, as shown in Table II).

Induction of CTL In a preliminary experiment for induction of allogeneic CTL from PBMC of a healthy volunteer against a gastric carcinoma cell line GT3TKB, the effect of different combinations of the interleukins was tested. We observed that CTL induced with the four interleukins (IL-1, -2, -4, and -6) showed stronger killing activity towards the target carcinoma cells than those induced with IL-2 alone, which is widely used in the traditional method for CTL generation (Fig. 1). At an E/T ratio of 1 in the assay course of 24 h, the addition of more interleukins (IL-1, -4 and -6) to the induction culture produced more active CTL. Therefore, the IL-cocktail of IL-1, -2, -4, and -6 was used in the following experiments on induction of autologous CTL against tumor cells.

In the present protocol for induction of autologous CTL with the IL cocktail, lymphocytes in the PBMC aggregated on the carcinoma cells within 3–5 days and began to proliferate. The target carcinoma cells disappeared completely after 7–10 days. After most of the non-proliferating PBMC initially added had died within 5–10 days, the lymphocytes began to burst (except Hpt.7 in Table I), usually after 10–14 days. This pattern of lymphocyte

Table I. Summary of Autologous CTL Culture

Patient	HLA-typing[a)]	Lymphocytes					
		Culture days	Initial no. of cells	Obtained no. of cells	$CD3^+$ (%)	$CD4^+$ (%)	$CD8^+$ (%)
Hpt.7	A25(34)	15	5.0×10^6	9.2×10^6	—	—	—
Hpt.9	A2, Bw4(A32)	39	2.0×10^6	1.0×10^7	—	—	—
Hp.10	A2, 2402, B8	15	2.2×10^6	5.0×10^7	99.8	5.7	94.2
Hpt.15	A2402	27	2.1×10^6	3.7×10^7	99.9	3.4	77.0
Hpt.16	A2, 11	41	2.0×10^6	4.3×10^7	93.1	89.4	7.0
Pt.45	A2402	46	3.3×10^6	4.5×10^7	—	—	—
Pt.46	A2402	50	2.5×10^6	3.2×10^8	99.9	64.4	35.5
TUHR3TKB	A2, 2402	49	5.0×10^6	4.7×10^{11}	98.2	33.2	66.7
TUHR4TKB	A2402, 25(38), Bw4(A32)	88	1.0×10^6	9.5×10^7	99.8	3.2	91.6
TUHR10TKB	A2402	19	3.2×10^6	2.8×10^7	97.2	1.9	80.1

a) HLA-typing was done as described in "Materials and Methods." Only HLA-A2402 was confirmed by the PCR method.

Table II. Killing Activity and Specificity of Autologous CTL

Patient	E/T ratio	Autologous targets		Allogeneic targets				
		Carcinoma	Normal	OS-RC-2	Hpt.10	Hpt.15	TUHR3TKB	TUHR4TKB
Hpt.7	4	29.9±2.7[a)]	135.3±10.2	294.7±2.2	126.0±13.6	95.6±7.7	139.3±5.7	193.1±17.3
Hpt.9	2	15.8±4.1	78.8±3.0	282.4±5.4	138.8±3.3	110.1±4.1	146.0±8.3	229.0±7.6
Hpt.10	2	32.9±2.9	ND	141.9±8.8	—	134.3±1.7	145.7±8.2	215.6±9.3
Hpt.15	2	–3.6±1.9	80.6±10.6	257.5±8.5	134.5±7.6	–3.6±1.9	144.8±5.9	260.8±22.9
Hpt.16	2	–1.3±1.4	ND	339.9±4.9	169.2±4.9	142.3±6.7	—	217.5±3.2
Pt.45	2	33.8±6.7	86.9±11.3	ND	ND	ND	ND	ND
Pt.46	2	10.3±8.4	95.9±13.9	199.0±5.6	43.1±11.6	167.8±7.3	150.0±13.4	199.3±15.4
TUHR3TKB	2	71.8±2.6	ND	342.6±15.7	84.9±8.8	ND	—	120.0±1.9
TUHR4TKB	2	137.8±1.9	ND	283.5±15.7	111.5±8.8	ND	62.4±3.9	—
TUHR10TKB	2	21.3±2.5	148.2±6.0	164.1±7.0	205.8±3.8	90.8±4.7	101.8±1.2	184.4±7.7

a) Killing activity was expressed as the percentage of surviving target cells remaining after the 24-h incubation. The A_{570} of each well of control target cells were separately measured at the start of the killing assay and this value was taken as 100% in calculation of the percentage of surviving target cells. Note that the target cells grew during the 24-h incubation period and therefore showed values higher than 100%. Each point shown is the mean of triplicate observations±SD. ND, not done.

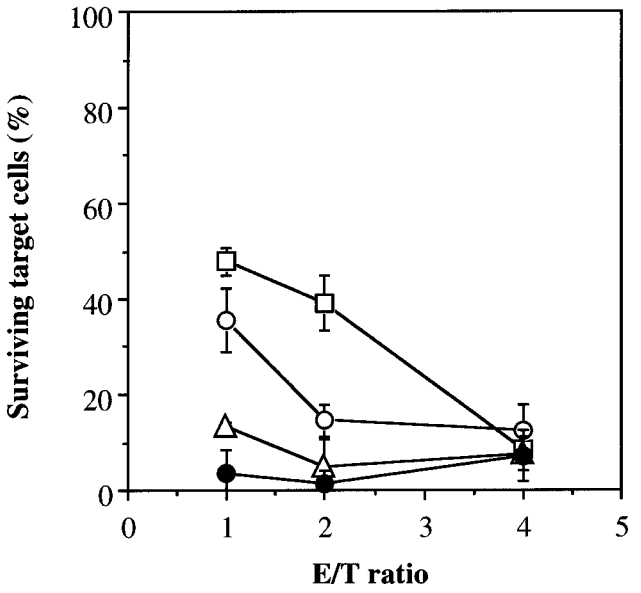

Fig. 1. Effect of the combination of interleukins on the cytotoxic activities of induced CTL. PBMC from a healthy volunteer (2×10^6 cells) were co-cultured with the allogeneic gastric carcinoma cells, GT3TKB, in the medium containing different interleukin combinations. The CTL were restimulated with the target cells up to 4 times. After 44 days, the CTL were submitted to cytotoxicity assay. The killing assay was performed for 24 h at the E/T ratios indicated. After the coculture of the effector cells with target carcinoma cells, the lymphocytes were gently washed out, then the adhering (therefore possibly the surviving) target carcinoma cells were fixed, stained with 0.4% crystal violet and quantified. CTL were induced with the medium containing IL-2 (□), IL-1 and -2 (○), IL-1, -2, and -4 (△), and IL-1, -2, -4, and -6 (●). Each bar shown is the mean of triplicate observations±SD.

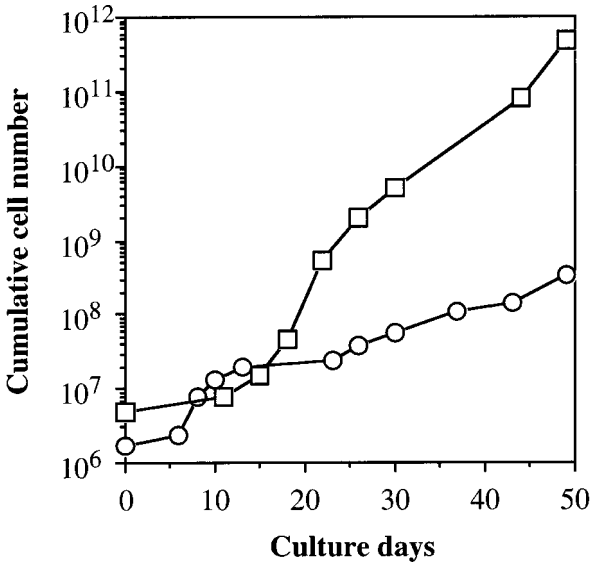

Fig. 2. Typical cumulative growth curves of autologous CTL induced against renal carcinoma cells. □ TUHR3TKB-CTL, ○ Pt.46-CTL.

growth was similar to that observed in the case of induction of allogeneic CTL.[21] The lymphocytes were restimulated twice with autologous carcinoma cells within the initial 2 weeks. Two typical cumulative growth curves of CTL are shown in Fig. 2, one with restimulation by autologous carcinoma cells (TUHR3TKB-CTL) and the other without antigen restimulation (Pt.46-CTL). The restimulation apparently boosted CTL growth from the initial 2×10^6 cells up to more than 10^{11} cells in 50 days of culture.

Table I summarizes the autologous CTL induction culture. The phenotypes of the lymphocytes were analyzed by flow cytometry except in the cases of Hpt.7, Hpt.9, and Pt.45, because of the shortage of generated lymphocytes. In most cases, the main population of lymphocytes consisted of $CD3^+CD8^+$ cells (Table I) except for the cells from patients Hpt.16 and Pt.46. In these cases, $CD4^+$ CTL became dominant after restimulation.

Killing activities of CTL Table II summarizes the killing activities and specificity of the autologous CTL. In 9 out of 10 cases, CTL have been induced. The CTL from patient Hpt.7 lysed the autologous target carcinoma cells at an E/T ratio of 4 in the course of 24 h. Other CTL examined at an E/T ratio of 2 also lysed autologous target cells except in the case of patient TUHR4TKB. The CTL from patients Hpt.15 and Hpt.16 completely lysed autologous target carcinoma cells at this low E/T ratio. However, none of the CTL vigorously killed autologous normal kidney epithelial cells. The CTL from patient TUHR3TKB showed only weak killing activity at this low E/T ratio, and we confirmed that this activity was reproducible. The CTL from patient Pt.46 killed not only autologous target carcinoma cells but also allogeneic Hpt.10 renal carcinoma cells. However, these CTL did not recognize the other renal carcinoma cells tested, OS-RC-2, Hpt.15, TUHR3TKB, and TUHR4TKB.

The lymphocytes cultured as CTL from patient TUHR4TKB did not lyse autologous target carcinoma cells, although the major population of cells in this preparation of CTL consisted of $CD8^+$ lymphocytes and these were partially effective in killing the allogeneic TUHR3TKB carcinoma cells. MHC class-I molecules (HLA-A2402) were undetectable on the TUHR4TKB target cell surface (data not shown). In contrast, NK from the same patient (TUHR4TKB) lysed TUHR4TKB carcinoma cells and NK-sensitive K562 cells at an E/T ratio of 2 in the course of 48 h (Fig. 3), but these cells did not

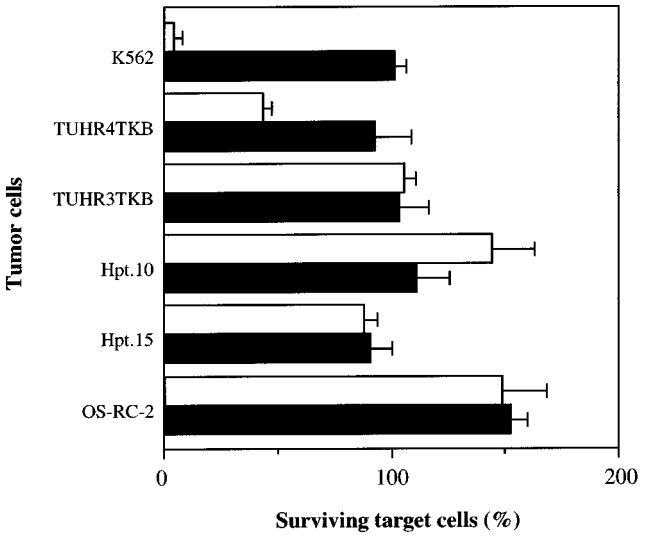

Fig. 3. Cytotoxicity of NK cells towards renal cell carcinomas. Target tumor cells (1×10^4 cells/well) and NK cells ($CD56^+$ cells were more than 95%) were co-cultured for 48 h at an E/T ratio of 2. The surviving tumor cells were measured as described in "Materials and Methods." Note that A_{570} of the target carcinoma cells at the start of the co-culture was taken as 100%. Also note that the carcinoma cells cultured at the E/T ratio of 0 grew during the 24-h incubation and, therefore, showed more than 100% survival. Each bar shown is the mean of triplicate observations±SD. □ with NK cells, ■ without NK cells.

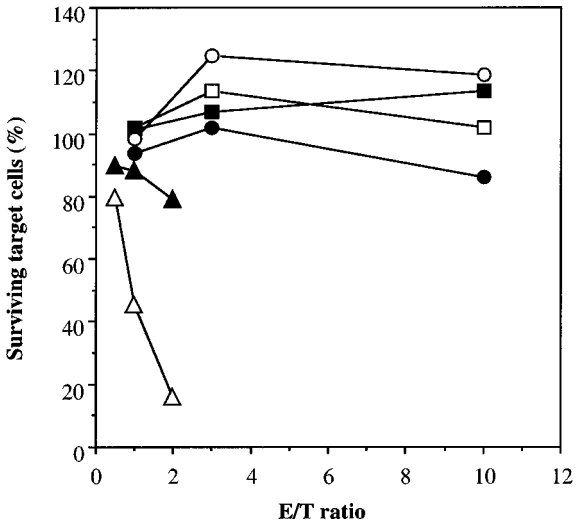

Fig. 4. A comparison of the activity of autologous effector lymphocytes derived from the patient Hpt.9. The target tumor cells and the effector cells were co-cultured for 48 h. □ NK on tumor cells, ■ NK on normal cells, ○ LAK on tumor cells, ● LAK on normal cells, △ CTL on tumor cells, ▲ CTL on normal cells.

lyse other MHC class-I-expressing allogeneic tumor cell lines, TUHR3TKB, Hpt.10, Hpt.15, and OS-RC-2. These data are consistent with the mechanisms of NK recognition and killing.[28)]

The CTL from patient Hpt.9 showed more than 10-fold greater killing activity than autologous LAK and NK (Fig. 4). In other patients, the NK and LAK showed little or no killing activity at an E/T ratio of 10 against autologous carcinoma cells (data not shown).

DISCUSSION

The present results suggest that CTL are inducible in almost all cases of cultured renal carcinoma cells displaying MHC class-I expression. The CTL showed autologous target-specific cytotoxicity, although the specificity was weak in two cases, Pt.46-CTL against Hpt.10 carcinoma cells and TUHR4TKB-CTL against TUHR3TKB carcinoma cells (Table II). The cytotoxicity was inhibited by previous treatment of the CTL with anti-CD3 antibody (data not shown). No evident cytotoxicity against normal renal epithelial cells was observed in the 6 cases examined so far, suggesting that the antigens might not be expressed on normal cells. This situation is different from the case of melanoma patients, where the CTL generated from TIL effective against autologous melanoma cells also lysed normal melanocytes.[1, 5)] It is possible that the TIL had been primed to differentiate into CTL because of continuous contact with tumor cells *in vivo*. Therefore, CTL should be generated from TIL in the case of all melanoma patients. However, although tumor-specific CTL could be isolated from 9 of 24 cases through the culture of TIL with repeated stimulation by autologous melanoma cells,[29)] effective lymphocytes could not be generated in 62.5% of the 24 cases.

The generation of highly active CTL and the 90% success in induction of CTL against MHC class-I-expressing renal carcinoma cells may possibly be ascribed to the use of the cocktail of IL-1, -2, -4, and -6, while most researchers have used only IL-2 throughout the entire culture process. IL-6 and IL-1 accelerate the development of cytotoxicity,[30, 31)] and IL-4 mediates the functional differentiation of $CD8^+$ T cells.[31)] The combined cocktail must have cooperatively promoted the process of induction of CTL. In the case of patient Hpt.16, the generation of $CD4^+$ CTL was evident (Table I). The killing activity of these cells was as strong and as specific for autologous carcinoma as that of the $CD8^+$ CTL from patient Hpt.15. At present, we have no evidence to explain why $CD4^+$ CTL were preferentially generated in this patient. Since the cross reactivity of CTL of Hpt.10, Hpt.15, Pt.46 TUHR3TKB, and TUHR10TKB against allogeneic targets

expressing common HLA-A2402 was generally low (Table II), unique antigens might have been expressed on the renal carcinoma cells from each case.

The present results suggest that there are many immature lymphocytes in the peripheral circulation that might be educated to attack autologous renal carcinoma cells. To provide a sufficient amount of CTL, the present induction technique of CTL can be combined with the expansion techinique of T lymphocytes using anti-CD3 antibody stimulation[21] to overcome a possible shortage of autologous target cells for restimulation in the CTL culture. If there are any allogeneic tumor cells expressing common renal carcinoma antigens on their cell surface, they may also be useful. These findings may contribute to adoptive immunotherapy of renal cancer patients in which expression of MHC class-I molecules is detected on the carcinoma cells.

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