Recycled addition of CD4⁺ T cell-rich population for induction of human autologous cytotoxic T lymphocytes: A practically efficient method

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Abstract

When CD4⁺ T cell-rich population appears in the initial trial in induction cultures of human autologous cytotoxic T lymphocytes (CTL), the culture results frequently in no or weak killing activity and therefore usually be discarded as an 'unsuccessful' CTL induction culture. However, addition of the initial CD4⁺ T cell-rich population enabled efficient induction of the autologous CTL in the ensuing trials. The CTL thus generated exhibited stronger killing activities against autologous brain tumor cells and ovarian tumor cells than previously observed. This simple recycling of the primed but inert CD4⁺ T cell-rich population for CTL induction will promote clinical practice of adoptive immunotherapy of human tumors with autologous CTL.

Abbreviations: CTL, cytotoxic T lymphocytes; IL, interleukin; LAK, lymphokine-activated killer cells; NK, natural killer cells; TIL, tumor-infiltrating lymphocytes

Introduction

Recent development of the culture method of human lymphocytes (Kawai et al., 1992; Liu et al., 1995) enabled us to induce autologous cytotoxic T lymphocytes (CTL) with strong killing activity when the combination of interleukin(IL)s of IL-1, -2, -4, and -6 was applied (Liu et al., 1996; Tsurushima et al., 1996). Especially against renal carcinoma cells, autologous CTL were inducible from 9 out of 10 (Liu et al., 1998).

However, with other malignant tumors such as those from brain, the rate of successful induction of autologous CTL is not necessarily as high as with renal cancers (our unpublished data). In the course of CTL induction from peripheral blood mononuclear cells (PBMC), we have experienced many cases in which lymphocytes expressing CD4 surface molecules grew fast and predominated the cultured lymphocyte

population, but the cells did not exhibit any killing activity against the autologous target tumor cells.

These CD4⁺ T cells, usually discarded as a result of 'unsuccessful' CTL induction not only in our laboratory but also in other laboratories, may contain Th1-type helper cells and be useful for the generation of autologous CTL carrying strong killing activity (Stuhler and Walden, 1993; Stuhler and Schlossman, 1997; Bennett et al., 1997; Goedegebuure et al., 1997; Ostankovitch et al., 1997; Ossendorf et al., 1998). However, the purification and re-addition process of autologous Th1 cells is tedious, and, for practical clinical use in adoptive immunotherapy of tumors, a simple and efficient method has not been reported for generation of human autologous CTL. Here we describe that the simple recycled addition of the CD4⁺ T cell-rich population, which appeared in the initial trial for the autologous CTL induction, enabled efficient

generation in the ensuing trials of human autologous CTL with high killing activity of autologous tumor cells.

Materials and methods

Reagents, cell lines, and blood lymphocytes

Recombinant human IL-1 β , -2, -4, and -6 were provided by Ohtsuka Pharmaceutical Co., Ltd. (Tokushima), Shionogi & Co., Ltd (Osaka), Genzyme, Co. (Tokyo), and Ajinomoto, Inc. (Kawasaki), respectively. RHAM α medium (Kawai et al., 1992) was provided from the routine pool for media in RIKEN Cell Bank and used for culture of human lymphocytes. Antibodies were purchased as follows: FITC-labeled goat anti-mouse IgG polyclonal antibody, Becton-Dickinson, Co.; anti-CD3, anti-CD4, and anti-CD8 monoclonal antibodies, Nichirei Co., Tokyo; anti-human MHC-class I monoclonal antibody (W32/64 clone), Dako Japan Inc.; anti-human MHC-class II monoclonal antibody (CR3/43 clone), Dako Japan Inc.

Human materials used in the present study were all supplied from University of Tsukuba Hospital after obtaining the informed consent from each patient. The target tumor cells used were all provided from the routine primary cultures running in RIKEN Cell Bank as has been described for renal carcinoma cells (Liu et al., 1998). Human PBMC were prepared by the conventional Ficoll-Paque centrifugation method from heparinized peripheral blood of the tumor patients. The cells were washed once with PBS, then once with RHAM α medium supplemented with 5% autologous plasma and centrifuged at 1,400 rpm (260 g) for 10 min at room temperature.

Induction of CTL

Before addition of the PBMC for CTL induction, confluent target tumor cells maintained in a 24-well plate were irradiated with 50 Gy of X-rays. The PBMC (1×10^6 cells/ml, 1 ml/well) were then cultured on these autologous tumor cells (at this stage, the responder:stimulator ratio was approximately 10) in the induction medium, i. e., RHAM α medium supplemented just before use with 5% autologous plasma and the IL-cocktail [IL-1 β (167 U/ml), IL-2 (67 U/ml), IL-4 (67 U/ml), and IL-6 (134 U/ml)] (Liu et al., 1996; Tsurushima et al., 1996).

The CTL induction culture was continued with appropriate changes of this medium including the IL-

cocktail (at least half of the medium was changed every 2 days) until the target cells disappeared almost completely. For re-stimulation, the lymphocytes adjusted at 10^6 cells/ml in the induction medium were transferred onto irradiated target tumor cells at an E/T ratio of 10 after 13–22 days in the primary induction culture. The timing of re-stimulation was determined on the disappearance of almost all the target. Within a day after the transfer, most of the target cells were killed. This was assigned as RS1 (the first re-stimulation). Then, 7–10 days later, RS2 (the second re-stimulation) was conducted as for RS1 if growth of the lymphocytes turned out to be apparently slow under microscopic observation.

In case that the above cultured lymphocyte population just before RS1, or after the RS1, killed scarcely the target cells and found to be CD4⁺ T cell rich, the lymphocytes were used for the recycling in the ensuing CTL induction culture after irradiated with 30 Gy of X-rays. These lymphocytes (1×10^5 cells) were added to 1×10^6 fresh PBMC. Then the ensuing CTL induction was performed just as described above.

Flow cytometry and HLA-typing

The cultured lymphocytes were stained with FITC- or PE-labeled antibodies (against human CD3, CD4, CD8, CD40L, CD56, MHC class-I, and MHC class-II) according to the manufacturer's protocol. The stained cells were analyzed by flow cytometry in a FAC-Scan with LYSIS II software (Becton Dickinson, CA, USA).

General HLA typing was performed using a Terasaki Oriental HLA-ABC Tray (ONE LAMBDA, Inc., California). HLA-A24-subtypes were determined by PCR with the HLA-A allele-specific primer sets (Blasczyk et al., 1995).

Killing assay

Cytotoxic activity of CTL was determined as described (Liu et al., 1995) by co-culturing lymphocytes and the target tumor cells for 24 h. The target cells adhering to the bottom of the wells were measured after the crystal violet staining (see Methods in the reference, Liu et al., 1995). The cells remaining in the wells of the assay plate were expressed as % surviving cells. For lymphocytes which are strongly cytotoxic, this assay is as sensitive and reliable as the standard ⁵¹Cr-release assay for assessment of the killing activity when it is used with adherent target cells at effector/target (E/T) ratios less than 10 (see Figure 1

Figure 1. Killing activities of autologous CTL with or without induction by the addition of recycled autologous CD4⁺ T cell-rich population that was generated in the initial trial of the induction culture in each patient. A radio-isotope-free method for determination of the killing activity, namely crystal violet staining, was adopted in the present study (see Materials and Methods). Note that, to exclude the possibility of simple growth inhibition of the tumor cells by the CTL during the 24-h coculture, we separately measured the 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. Some tumor cells at the E/T of 0 grew during the 24-h incubation and therefore showed values higher than 100%. Each point shown is the mean of triplicate observations. (a) AiTo-CTL: PBMC of patient AiTo with a brain tumor (glioblastoma multiforme, GBM) were cultured for induction of the autologous CTL (see text) with (●) or without (□) the CD4⁺ T cells (see Table 1). Killing assays were performed at day 15 before the re-stimulation with the target cells (upper figure), at day 25 after the first re-stimulation with the target cells (RS1) made at day 15 (middle figure), and at day 33 after the second re-stimulation with the target cells (RS2) made at day 25 (lower figure). (b) Opt.46-CTL: PBMC of patient Opt.46 with an ovarian carcinoma were cultured for induction of the autologous CTL with (●) or without (□) the unpurified CD4⁺ T cell-rich population. Killing assays were performed at day 13 just before RS1 (upper figure), and at day 22 after RS1 made at day 13 (lower figure). (c) MaSu-CTL: PBMC of patient MaSu with GBM were cultured for induction of the autologous CTL with (●) or without (□) the CD4⁺ T cells. Killing assays were performed at day 14 just before RS1 (upper figure), and at day 22 after RS1 made at day 14 (lower figure).

of the reference, Liu et al., 1995). To exclude the possibility of simple growth inhibition of the tumor cells by the CTL during the coculture, we separately measured the A570 of wells of the 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 target tumor cells at the E/T ratio 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.

Results

Phenotypes of the lymphocytes after the initial trials in the induction cultures of autologous CTL

Table 1 shows phenotypes of the lymphocytes that were used for the recycling in the ensuing CTL induction cultures. These CD4⁺ T cell-rich populations were products of the initial trials in the induction cultures of autologous CTL from PBMC of the tumor-bearing patients. If the PBMC from the patients contain a sufficient number of precursors of the CTL and their target cells are exhibiting a level of antigenicity

Table 1. Phenotypes of CD4⁺ T cell-rich population recycled for induction of autologous CTL from fresh PBMC

Patients	Target tumor cells	Phenotype of CD4 ⁺ T cell-rich population used for the recycled addition			Corresponding Figure No.
		CD3 ⁺	CD4 ⁺	CD8 ⁺	
AiTo	GBM**	98% <	98% <	0%	Fig. 1a
Opt.46	ovarian carcinoma	90%	66%	21%	Fig. 1b
MaSu, 1st*	GBM	98% <	98% <	0%	Fig. 1c
MaSu, 2nd	GBM	98% <	98% <	0%	Fig. 2a
MaSu, 3rd	GBM	98% <	98% <	0%	Fig. 2b
HiTo, 1st	GBM	–	–	–	Fig. 3
HiTo, 2nd	GBM	98% <	96%	3%	Fig. 4
NaKa	GBM	97%	86%	11%	Fig. 5

* When plural trials of CTL induction were carried out parallel for the same patient, the CTL were named as MaSu-CTL-1, MaSu-CTL-2, etc.

** GBM, glioblastoma multiforme.

similar to the tumors, the cultured lymphocytes are expected to exhibit killing activity against their autologous target tumor cells as previously described (Liu et al., 1998).

We have defined successful autologous CTL induction, namely, that in the killing assay the lymphocytes should reduce the number of live target tumor cells to less than 50% at an E/T ratio of 4 within 24 hr. According to this definition, PBMC of patients bearing astrocytoma-grade III or glioblastoma multiforme (GBM) were not necessarily able to generate CTL. So far as we have tried to induce their autologous CTL, the success rate was 40% (4 out of 10 patients).

When we tried to induce CTL from patient AiTo who had been suffering from recurrent GBM, his PBMC did not generate CTL even after the second re-stimulation performed at day 25 in the culture. His CTL, if induced, had been expected to kill most of the autologous target tumor cells at an E/T ratio of 8 within 24 hr. However, the density of the target cells increased in the killing assay when the E/T ratio was increased (Figure 1a, Day 33, open squares). This patient's PBMC became almost pure CD4⁺ T cell population (Table 1, AiTo) carrying no killing activity.

Efficient induction of autologous CTL after the recycled addition of CD4⁺ T cell-rich population

After irradiation with 30 Gy of X-rays, the CD4⁺ T cell population from patient AiTo was mixed with his own fresh PBMC at a ratio of 1:10 and induction of autologous CTL was tried. The irradiation before the mixing was necessary to suppress possible over-

growth of CD4⁺ T cells in the ensuing trials. This recycled addition of the CD4⁺ T cells to the induction culture generated AiTo-CTL with apparent killing activity against autologous tumor cells at the E/T ratio of 8 as early as day 15 (Figure 1a, Day 15, closed circles). At this E/T ratio, the CTL reduced the surviving target tumor cells to 45% of the initial number of the tumor cells that was defined as 100% in the killing assay. After RS1 at day 15, the AiTo-CTL population at day 25 killed more tumor cells and reduced the surviving target cells to 67% at an E/T ratio of 4 (Figure 1a, Day 25, closed circles). This population assumed stronger killing activity after the RS2 at day 25. The surviving target tumor cells were reduced to 38% at an E/T ratio of 2 (Figure 1a, Day 33, closed circles).

Improved killing activities were observed in induction cultures of autologous CTL from other patients by the simple mixing of irradiated autologous CD4⁺ T cell-rich population with fresh PBMC (Figure 1b, and c, closed circles). In the case of patient Opt.46, killer lymphocytes exhibiting considerable activity against the autologous ovarian carcinoma cells were induced after day 13 in the control culture without addition of CD4⁺ T cell population (Figure 1b, Day 13). However, when the PBMC were cultured with the recycled autologous CD4⁺ T cell-rich population of which CD4⁺ T cells comprised 66% (Table 1), the killing activity of Opt.46-CTL was apparently enhanced as seen with an E/T ratio of 2 or less (Figure 1b, day 13 and day 22).

Enhancement of killing activity of CTL induced after recycling of CD4⁺ T cells was also evident in

Figure 2. Specific killing activity of MaSu-CTL-2 against autologous target tumor cells. (a) MaSu-CTL-2 killed the autologous GBM cells (●) but not the allogeneic AiTo GBM cells (□). (b) Inhibition of the killing activity by antibodies against the surface molecules. Anti-CD3, -CD8, and -MHC class I inhibited the killing activity.

the assay of MaSu-CTL-1 (Figure 1c, Day 14, closed circles). The control CTL (conventionally generated without addition of the autologous CD4⁺ T cells) showed killing activity at day 14; 50% of the initial autologous GBM cells were killed at the E/T ratio of 8, although the target cell killing was not apparent at an E/T ratio of 2. However, the MaSu-CTL-1 induced with the autologous CD4⁺ T cells killed most of the target tumor cells at the low E/T ratio of 2 (Figure 1c, Day 14, closed circles). The enhancement of the killing activity was not so apparent at day 22 after RS1 conducted at day 14, since strong killing activity was also observed in the control CTL (Figure 1c, Day 22).

By use of flow cytometry, the proportion of CD8⁺ T cells in the AiTo-CTL induced with the recycled autologous CD4⁺ T cells was observed to decrease rapidly from 32% to 10% and 6% after RS1 and RS2, respectively (Table 2). Conversely, Opt.46-CTL induced with the autologous CD4⁺ T cell-rich population and re-stimulated once with the autologous target cells showed that CD8⁺ T cells comprised 92% of the population at day 22, up from 71% prior to RS1 (Table 2). MaSu-CTL-1 (the first CTL population from patient MaSu) induced with the autologous CD4⁺ T cells contained 48% of CD8⁺ T cells at day 22 after RS1, up from 20% prior to RS1 (Table 2). The percentage was close to that of control MaSu-CTL-1 generated without the addition of CD4⁺ T cells.

Specific killing activity of the induced CTL

Figure 2a shows the specific killing activity of MaSu-CTL-2 (the second CTL population induced with the autologous CD4⁺ T cells from the same patient MaSu) against the autologous brain tumor cells (closed circles); they were relatively ineffective against the allogeneic brain tumor cells from patient AiTo (open squares). These two patients carry HLA-A2, A24, B52, B62(B75), -/-, and HLA-A11, B62, B54, Cw1, Cw4, respectively.

Since MaSu-CTL-1 induced with autologous CD4⁺ T cells comprised 49% of CD4⁺ T cells and MaSu-CTL-2 contained 32% of CD4⁺ T cells (Table 2), we examined whether cytotoxic activity of CTL generated from this patient was derived from CD8⁺ killer T cells. The killing activity of MaSu-CTL-3 (the third CTL population induced with autologous CD4⁺ T cells from patient MaSu) containing 45% and 55% of CD4⁺ and CD8⁺ T cells, respectively (Table 2), was apparently inhibited by the pretreatment with monoclonal antibodies against CD3, CD8, and MHC-class I (Figure 2b) but not with those against CD4 and MHC class-II molecules.

Growth of the CTL

Ridge et al. (1998), Bennett et al. (1998), and Schoenberger et al. (1998) demonstrated independently that helper T cells act on antigen-presenting cells (APC) through binding of CD40 ligand (CD40L) on their surface to CD40 on APC and thereby increasing IL-12 release from APC. The cytokine release is expected to contribute to induction of CTL.

In a preliminary experiment, PBMC of patient HiTo bearing GBM could not generate active CTL in

Table 2. Phenotypes of generated CTL population

CTL	induction	RS*	Determined at day	Phenotype			
				CD56 ⁺	CD3 ⁺	CD4 ⁺	CD8 ⁺
AiTo-CTL-1	without CD4**	0	15		89%	64%	21%
	with CD4	0	15		74%	37%	32%
	without CD4	1	25		98% <	92%	0%
	with CD4	1	25		94%	91%	10%***
	without CD4	2	33		98% <	91%	1%
	with CD4	2	33		98% <	94%	6%
Opt.46-CTL	without CD4	0	13		44%	67%	17%
	with CD4	0	13		90%	7%	71%
	without CD4	1	22		97%	72%	24%
	with CD4	1	22		98%	25%	92%
MaSu-CTL-1	without CD4	0	14		98%	88%	9%
	with CD4	0	14		91%	49%	20%
	without CD4	1	22		78%	47%	45%
	with CD4	1	22		91%	26%	48%
MaSu-CTL-2	with CD4	1			98% <	32%	78%
MaSu-CTL-3	with CD4	1			98% <	45%	55%
HiTo-CTL-1	IL-1,2,4,6#	0	14	0%##	98% <	96%	2%
	+ IL-12	0	14	0%	93%	93%	3%
	+ IL-12 (day 0,7)	0	14	1%	86%	64%	28%
HiTo-CTL-2	without CD4	1	20	0%	98% <	97%	2%
	with CD4	1	20	0%	98% <	80%	17%
NaKa-CTL	without CD4	1	20	3%	93%	47%	56%
	with CD4	1	20	2%	95%	71%	62%

* RS, re-stimulation of the cultured lymphocytes with the autologous target tumor cells.

** CD4, autologous CD4⁺ T cell-rich population.

*** Some of the population contained CD4⁺ CD8⁺ (double positive) T cells, that resulted in the percent of (CD4⁺ plus CD8⁺) being greater than that of CD3⁺.

IL-1, -2, -4, -6 were added regularly in the induction culture without the CD4⁺ T cell-rich population.

Expression of CD56 was determined only for the lymphocytes in the CTL shown.

a culture containing the target autologous brain tumor cells and the IL cocktail (IL-1, -2, -4, -6). Then, induction of HiTo-CTL was attempted by addition of IL-12 together with the IL cocktail in the present culture medium. IL-12, continuously added or pulsed only at day 0 and day 7, stimulated the killing activity of HiTo-CTL-1 (Figure 3a) but reduced their growth rate (Figure 3b).

In another HiTo-CTL induction culture, recycled addition of the autologous CD4⁺ T cell-rich population resulted in an increased killing activity of HiTo-CTL-2 (Figure 4a) as has been seen in Figure 3a. The CD4⁺ T cell-rich population, just before the recycled addition, expressed CD40L (Figure 4b) but the growth of the lymphocytes in HiTo-CTL-2 induction culture was not inhibited when compared to the control HiTo-CTL-2 induction culture without addition of autologous CD4⁺ T cells (Figure 4c). For con-

firmation of the reproducibility of this phenomenon, we observed, by the recycled addition of the initial CD4⁺ T cell-rich population from patient NaKa bearing GBM (Table 1), the enhancement of cytotoxic activity of autologous CTL (Figure 5a), expression of CD-40L on the surface of the initial CD4⁺ T cell-rich population (Figure 5b), and no inhibitory influence of them on the growth of NaKa-CTL (Figure 5c).

CD56⁺, a marker for natural killer cells, has so far been studied in HiTo-CTL-1 and -2 and NaKa-CTL; CD56⁺ cells were seen to comprise 3% or less of the population (Table 2). The proportion of CD56⁺ cells was independent of the co-existence of autologous CD4⁺ T cell-rich population and the IL-12 supplementation in the induction culture.

Figure 3. Killing activities and growth of HiTo-CTL-1 modified with IL-12. CTL were induced from PBMC of patient HiTo bearing GBM in the induction medium containing the IL cocktail (□), the IL cocktail plus IL-12 (50 pg/ml) continuously (▲), and the IL cocktail plus IL-12 which was pulsed at day 0 for 24 h and day 7 for 24 h (△). Re-stimulation with the autologous target tumor cells was carried out at day 14. (a) Killing activities determined at day 22. (b) Growth curves of HiTo-CTL.

Discussion

The present experiments have demonstrated that recycling, but not discarding as a result of unsuccessful CTL induction, of the CD4⁺ T cell-rich population generated in the initial trial is a simple and efficient method for induction of human autologous CTL to a state of strong tumor cell killing activity. As has been described by many investigators (Stuhler and Walden, 1993; Stuhler and Schlossman, 1997; Bennett et al., 1997; Goedegebuure et al., 1997; Ostankovitch et al., 1997; Ossendorf et al., 1998), CD4⁺-expressing helper T cells, especially Th1-type, play an essential role in the induction of CTL through the interaction of their CD40L and CD40 on APC (Ridge et al., 1998; Bennett et al., 1998; Shoenberger et al., 1998). Therefore, it is reasonable to conclude that the CD4⁺ T cell-rich population includes autologous Th1 cells

Figure 4. Killing activity and growth of HiTo-CTL-2. The CTL were induced independently from the lot of HiTo-CTL-1 in the induction medium containing the cocktail of IL-1, -2, -4, -6 and with (●) or without (□) the recycled autologous CD4⁺ T cell-rich population. (a) Killing activity determined at day 20 after RS1. (b) Expression of CD40L on the surface of the recycled autologous CD4⁺ T cell-rich population (shaded area). Open peak shows unstained control cells. (c) Growth curves of the lymphocytes in the induction culture.

which were able to stimulate not only growth of the CTL but also killing activity (Figure 3). However, in the initial trial of CTL induction culture, PBMC from patient AiTo were not able to generate active CTL by

Figure 5. Characteristics of NaKa CTL. CTL were induced from PBMC of patient NaKa with (●) or without (□) the addition of the recycled autologous CD4⁺ T cell-rich population (see Table 1). (a) Their killing activities. (b) Expression of CD40L on the surface of the CD4⁺ T cell-rich population of patient NaKa (shaded area). Open peak shows unstained control cells. (c) Growth of NaKa-CTL induced with the recycled addition of the CD4⁺ T cell-rich population.

day 33 (Figure 1a), implying that CTL precursors in his PBMC had died before the Th1 cells grew.

Unexpectedly in the present experiments, we observed CD4⁺ T cell-rich CTL population from patients AiTo and HiTo after the re-stimulation with the

target tumor cells (Table 2). Although HiTo-CTL-2 induced with the CD4⁺ T cell-rich population contained 17% of CD8⁺ T cells, these CD8⁺ T cells may not have contributed to the killing of target tumor cells, since the HiTo-CTL-1 population taken from the same patient appeared to have contained only 3% CD8⁺ T cells (Table 2, line 18, +IL-12 population) but was accompanied with strong killing activity (Figure 3a, closed triangles). This population efficiently killed the target cells at an E/T ratio of 4, suggesting that the ratio of CD8⁺ T cells and target cells was 0.12. This low E/T ratio is insufficient to explain that the minor CD8⁺ T cells were real killers and killed almost all the target cells during the 24 hr incubation. Therefore, the population must have contained CD4⁺ T killer cells, although thoroughly purified CD4⁺ T cells should be examined to obtain the concrete result. Similarly, 2–3% CD56⁺ cells found in NaKa-CTL population (Table 2) may have not contributed to the killing activity in the assay shown in Figure 5a.

In a previous study, we have shown that human autologous CTL were efficiently inducible from 90% of renal carcinoma patients in the culture supplemented with the four IL cocktail (Liu et al., 1998). The high success rate of the CTL induction may possibly be a reflection of the relatively high immunogenicity of renal carcinomas, which has long been suspected from the fact that the incidence of spontaneous regression of renal cancers is considerably high (Challis and Stam, 1990; Freed et al., 1977). Under the same induction conditions, the success rate of CTL induction from GBM patients was 40%, suggesting that the four IL cocktail is not necessarily sufficient for CTL induction against relatively low immunogenic tumors.

To improve the success rate for the autologous CTL induction against brain tumor cells, we have tried supplementing the IL cocktail with IL-18 (Kohyama et al., 1998). IL-18 successfully induced autologous CTL from the PBMC which were non-responding to the four-IL cocktail. However, growth of the autologous CTL was not necessarily enhanced but rather reduced. Since IL-18 acts on the lymphocytes in quite a similar manner as IL-12 (Chouaib et al., 1994; Halverson et al., 1997), the growth inhibitory effect of IL-12 (Figure 3b) was predictable.

Other than the function of the helper T cells in stimulating antigen-presenting cells (APC) to release IL-12 (Macatonia et al., 1995; Scheicher et al., 1995; Cella et al., 1996; Heufler et al., 1996) that is inhibitory for the CTL growth, the helper T cells must have growth stimulatory potency to CTL precursors through

some mechanisms other than IL-12. This seems likely from the data shown in Figure 4c and Figure 5b. We, therefore, consider that recycling of the CD4⁺ T cell-rich population is advantageous to addition of IL-12 (or IL-18) for stimulation of the killing activity of CTL.

Further precise analyses, such as inhibition of the CTL induction with anti-IL-12 antibody, distribution of sub-population of the CD4⁺ T cells, and function of the molecules on the CD4⁺ T cells which may have stimulated directly the lymphocyte growth, were not possible, since we have adopted here a fully autologous system for the induction of human CTL. A shortage of PBMC and/or insufficient growth ability of the lymphocytes and target primary cultured tumor cells has hampered further analyses. However, the present fully autologous system for induction of CTL is extremely effective and is recommended for consideration of use in the clinical practice of adoptive immunotherapy of human tumors.

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