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Chang Hyun Kim · Takeshi Todoroki
Masatoshi Matsumura · Tadao Ohno

Eligibility of antigenic-peptide-pre-loaded and fixed adhesive peripheral blood cells for induction of cytotoxic T lymphocytes from cancer patients with elevated serum levels of carcinoembryonic antigen

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Abstract The inducibility of cytotoxic T lymphocytes (CTL) that react with carcinoembryonic antigen (CEA) was tested in cancer patients with elevated (more than 5 ng/ml) serum CEA levels when antigen presentation was carried out with paraformaldehyde-fixed adhesive peripheral blood mononuclear cells (PBMC) from the patient that had been pre-loaded with CEA652(9), an HLA-A2402-restricted tumor antigenic peptide derived from CEA. By culturing fresh autologous PBMC on the fixed cell layer in medium containing interleukin-1, -2, -4 and -6, three out of eight patients developed CTL. The CTL from two of these patients killed CEA-protein-producing gastric cancer cells carrying HLA-A2402 and the cells from the remaining patient killed CEA-non-producing stomach cancer cells pre-loaded with CEA652(9). The results suggest that a single antigenic peptide on the fixed adhesive cells will allow the ex vivo induction of peptide-reactive CTL that are easier to handle and allow antigen presentation without tedious preculture of the "professional" antigen-presenting dendritic-cells.

Key words Carcinoembryonic antigen · Cytotoxic T lymphocyte · HLA-A2402 · Epitope peptide · Immunotherapy

C. H. Kim · T. Ohno (✉)
RIKEN Cell Bank, RIKEN
(The Institute of Physical and Chemical Research),
Koyadai, Tsukuba Science City, 305-0074, Japan
e-mail: tad-ohno@rtc.riken.go.jp
Tel.: +81-298-36-9124
Fax: +81-298-36-9049

T. Todoroki
Department of Surgery, Institute of Clinical Medicine,
University of Tsukuba, Tennodai,
Tsukuba Science City, 305-0074, Japan

M. Matsumura
Institute of Applied Biochemistry
University of Tsukuba, Tennodai, Tsukuba Science City,
305-0074, Japan

Abbreviations *CEA* carcinoembryonic antigen · *CTL* cytotoxic T lymphocytes · *E/T ratio* effector to target ratio · *IL* interleukin · *MHC* major histocompatibility complex · *PBMC* peripheral blood mononuclear cells · *PBS* (−), calcium- and magnesium-free phosphate-buffered saline

Introduction

Cytotoxic T lymphocytes (CTL) play an essential role in cellular immunity in rejecting foreign invaders and tumors by recognizing an antigenic peptide in association with the major histocompatibility complex (MHC) (Unanue 1984; Babbit et al. 1985; Germain 1986; Yewdell and Bennink 1990). Previous reports have suggested that the ability of cells to present antigen and activate $CD8^+$ CTL can be further enhanced by increasing the concentration of epitope peptides nine or ten amino acids long that are complexed to MHC class I molecules expressed on the cell surface (Falk et al. 1991; Rammensee et al. 1993). The identification of tumor-associated antigen recognized by human cytotoxic T lymphocytes is being studied for immunotherapeutic benefit in the treatment of cancer. CTL can be induced against defined epitope peptides of a range of human tumor-associated antigens such as, for example, HER-2/neu (Fisk et al. 1995; Peoples et al. 1995), MART-1 (Kawakami et al. 1994), MAGE-1 (Traversari et al. 1992), MAGE-3 (Tanaka et al. 1997), CEA (Tsang et al. 1995), MUC-1 (Takahashi et al. 1994; Agrawal et al. 1996) and tyrosinase (Kang et al. 1995).

Carcinoembryonic antigen (CEA) is a well-known soluble tumor marker frequently detectable in peripheral blood of carcinoma patients. Elevated levels of CEA have been detected in the circulating blood of patients with many types of cancer (Muraro et al. 1985; Thompson et al. 1991). A recombinant vaccinia virus expressing CEA has been shown to be able to induce humoral and, simultaneously, cell-mediated anti-CEA immune responses in mice (Kantor et al. 1992). Previous

studies have shown that human dendritic cells pulsed with CEA-derived peptide or transfected with RNA elicited a peptide-specific CTL response by primary in vitro immunization in a culture system using peripheral blood mononuclear cells (PBMC) from carcinoma patients, though without record of the CEA level (Alters et al. 1997) or healthy donors (Alters et al. 1998; Nair et al. 1998; Kawashima et al. 1999; Nukaya et al. 1999). A 7-day pre-culture in medium containing cytokines was prerequisite for the preparation of dendritic cells, which are unable to proliferate and therefore must be repeatedly prepared from fresh PBMC from the donor for weekly re-stimulation in the CTL induction culture (Kawashima et al. 1999; Nukaya et al. 1999).

We have reported that CEA-specific CTL were generated by culturing healthy human PBMC. However, the CTL induction was carried out on formalin-fixed autologous adhesive PBMC that had been loaded with CEA protein bound to latex beads (Kim et al. 1998). The fixation of the antigen-presenting cells facilitated re-stimulation during the CTL induction. The resulting CTL could kill CEA-producing carcinoma cells and efficiently recognized the epitope peptides. We also showed induction of healthy donor CTL on the autologous adherent cells pulsed with a CEA-derived peptide in vitro.

On the basis of these results, we tried to induce peptide-reactive CTL from PBMC of carcinoma patients showing elevated serum CEA levels who may have developed a high level of tolerance or anergy (Staveley-O'Carroll et al. 1998) to CEA-producing cells. The results suggest that our method is able to induce CTL from the PBMC of carcinoma patients without pre-culture of dendritic cells.

Materials and methods

Target cells, culture condition, and HLA typing

Two cell lines with the same MHC class I phenotype, HLA-A2402/B5201/Cw1202, were selected, namely, a gastric adenocarcinoma cell line MKN45 that is poorly differentiating and well known to produce a high level of CEA (Hareyama et al. 1991) and, as a control, a stomach adenocarcinoma cell line GT3TKB that does not produce CEA. These cell lines were provided by the RIKEN Cell Bank and maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) in an atmosphere of humidified 5% CO_2 in air. It had been confirmed that the cell lines expressed MHC class I molecules on their surface and carried genes for the same MHC class I antigens, i.e., HLA-A2402, B5201, Cw1202. The HLA type was determined by the method of Blasczyk et al. (1995). The HLA phenotyping of each patient was performed using a Terasaki oriental HLA-ABC well tray (One Lambda Inc, Canoga Park, Calif.).

Peptides and reagents

The HLA-A24-restricted CEA peptide (amino acids 652–660, TYACFVSNL) and a control irrelevant peptide (amino acids 38–47, REYIQMCTEL) were purchased from Biotech Research Lab., Takara Shuzo Co. Ltd., Ohtsu, Japan. Sequences of the CEA peptides were derived from the CEA precursor (Swiss Prot: P06731). The control irrelevant peptide was derived from a nucleoprotein of the influenza virus (Swiss Prot: P18277). Fluorescein-isothiocyanate-labeled goat anti-(mouse IgG) polyclonal antibody was purchased from Becton-Dickinson Co., anti-CD3, anti-CD56, anti-CD4, and anti-CD8 monoclonal antibodies from Nichirei Co., Tokyo; and anti-(human MHC class I) monoclonal antibody (w6/32 clone) and anti-(human MHC class II) monoclonal antibody (CR3/43 clone) from Dako Japan Inc.

Preparation of antigen-presenting cells and pulsing with CEA peptide

With the ethical approval of the University of Tsukuba Hospital and after informed consent had been given, human PBMC were prepared by the conventional Ficoll-Paque centrifugation method from heparinized peripheral blood of each patient bearing a CEA-expressing carcinoma who had been identified, first by a serological method and then, whenever required, by genotyping, as carrying the HLA-A2402 subtype. The cells were washed once with calcium- and magnesium-free phosphate-buffered saline [PBS(−)] then once with RHAMα medium supplemented with 5% autologous plasma and centrifuged at 1400 rpm for 10 min at room temperature as previously described (Kim et al. 1998). The separated PBMC (2×10^6 cells) were allowed to adhere to a well of 24-well culture plates for 2 h at 37 °C in RPMI-1640 medium containing 10% FBS. The adherent cells were further cultured in the 24-well plate with 2 ml medium containing CEA peptide (50 μg). After 2 h in the culture, the adherent cells were fixed with 3% paraformaldehyde in PBS for 1 h at room temperature and washed thoroughly with PBS(−) and the culture medium overnight at 37 °C to remove the remaining paraformaldehyde completely before the induction of CTL. These fixed adherent cells were preserved at 4 °C until use.

Induction of antigen-specific CTL from PBMC

To the fixed adherent cells, freshly prepared PBMC (1×10^6 cells) from the same carcinoma patient were added with 2 ml RHAMα medium supplemented with human interleukin(IL)-1β (Otsuka Pharmaceutical Co. Ltd., 167 U/ml), IL-2 (Shionogi & Co. Ltd., 67 U/ml), IL-4 (Genzyme Co. Ltd., 67 U/ml), IL-6 (Ajinomoto, Co. Inc., 134 U/ml), and 5%(v/v) autologous plasma. The induction culture was continued for 4 weeks, half the medium being changed every other day. The effector cells were re-stimulated weekly to a total of three re-stimulations at an effector/adherent-cell ratio of 2 on the fixed adherent cells.

Cytotoxicity assay

The assay for cell-mediated killing of target cancer cells was performed in vitro by the crystal violet staining method as described previously (Liu et al. 1995). Briefly, the target cells, 1×10^4 cells/well in 200 μl RPMI-1640 medium containing 5% FBS, were seeded in each well of 96-well plates and were pre-cultured for 12 h. The cultured target cells were washed once with PBS(−), then the cultured PBMC suspended in 200 μl RHAMα medium containing 5% autologous plasma were added to each well as effector cells at the indicated effector to target (E/T) ratio. The cells were cocultured for 24 h. The wells were then washed once gently with the appropriate amount of calcium- and magnesium-containing PBS. Adherent target cells were fixed for 1 h with 10% (v/v) formalin (100 μl/well), then stained with crystal violet solution (0.4% in water, 80 μl/well) for 30 min at room temperature. The plate was washed with tap water and dried at room temperature. To each well, 100 μl 80% methanol was added and the A_{570} of each well was determined. As 100% control, the A_{570} of the target cells pre-cultured in a separate plate was determined just before the addition of the effector cells.

The percentage of surviving target cells was defined as follows:

$$\text{Surviving target cells } (\%) = [(A - C)/(B - C)] \times 100$$

where A is the A_{570} of the well containing the target cells and the CTL, B is the A_{570} of the 100% control well of the target cells, and C is the A_{570} of the well containing medium alone.

In the present experiments, the wells containing the CTL alone showed almost the same A_{570} as the medium alone, even in the wells corresponding to an E/T of 10, indicating that the lymphocytes remaining in the wells after the 24-h coculture had been sufficiently washed-out. A small proportion of the lymphocytes may have remained attached to the target cell layer, but they were included as surviving target cells. Therefore, the killing activity of the CTL should be considered to be slightly under estimated in this assay. Each value in the figures represents the mean of four replicates accompanied by an error bar representing the SD. Note that the target carcinoma cells cultured at E/T = 0 grew rapidly over the 24-h incubation and, therefore, showed more than 100% survival.

For testing CTL recognition of the HLA-A24-binding peptide derived from CEA, GT3TKB target cells were incubated with CEA652(9) at a final concentration of 50 μg/ml for 2 h at 37 °C and washed once with the culture medium; the effector cells were then added and cocultured for 24 h. To detect activities of natural killer cells or lymphokine-activated killer cells, a standard europium-release assay was performed in the 4-h coculture of the effector lymphocytes and the target K562 cells and Daudi cells as described (Volgmann et al. 1989).

Flow cytometry

The lymphocytes (1×10^6) were washed three times with PBS (−), stained for 30 min with the monoclonal antibodies, washed with PBS (−), and then stained for 30 min with FITC-labeled goat anti-(mouse IgG) polyclonal antibody. The cells were again washed with PBS(−) containing 4% FBS. They were resuspended in the same buffer at a concentration of 1×10^6 cells/ml and were immediately analyzed by fluorescence-activated cell sorting (FACScan; Becton-Dickinson) as previously described (Liu et al. 1995). The proportion of $CD3^+$, $CD56^+$, $CD4^+$, $CD8^+$ cells was determined using the corresponding monoclonal antibodies.

Inhibition of the cytotoxic activity of the cultured lymphocytes with monoclonal antibodies

Effector cells were pretreated with monoclonal antibodies against CD3, CD8, or CD4 as described previously (Liu et al. 1995). These antibodies were used at a final concentration of 20 μg/ml. Target MKN45 cells were precultured overnight in 96-well plates at 1×10^4 cells/well. These target cells were also pretreated as described (Liu et al. 1995) with antibodies against human MHC class I and MHC class II at a final concentration of 16.5 μg/ml and 20.3 μg/ml respectively, and then incubated with the effector cells at 37 °C for 6 h. The adherent target cells were quantified by the crystal violet staining method (Liu et al. 1995). The inhibition of cytotoxic activity was calculated according to following formula:

$$\text{Inhibition } (\%) = [(A - B)/(T - B)] \times 100$$

where A is the A_{570} value of the target cells to which the effector cells had been added, and where the target cells or the effector cells had been pretreated with a monoclonal antibody, B is the A_{570} value of the target cells to which the effector cells had been added but neither cell type had been pretreated with any monoclonal antibodies, and T is the A_{570} value of the target cells to which neither CTL nor antibodies had been added.

Enzyme-linked immunosorbent assay (ELISA) of IFNγ and TNFα

For determination of interferon γ (IFNγ) and tumor necrosis factor α (TNFα) production, supernatants of the effector cells exposed for 24 h to peptide-pulsed and fixed adherent cells at a responder:stimulator ratio of 2 were submitted to ELISA according to the manufacturer's protocol (PharMingen, San Diego. Calif.). The results were expressed in picograms per milliliter.

Results

Selection of patients and the target cell lines for the killing assay

Patients expressing HLA-A2402 on the surface of their cells were the subjects of the present study, since approximately 60% of Japanese carry the HLA-A24 gene and more than 99% of the HLA-A24 alleles are known to be HLA-A2402 (Tokyo Laboratory, Shionogi Biochemicals, personal communication). This high percentage of the HLA-A2402 allele allowed us to omit further confirmation by genotyping in the selected patients, who were determined by serotyping to carry HLA-A24. Since the threshold level of serum CEA has been set at 5 ng/ml by the University of Tsukuba Hospital, patients for the present study were selected whose serum CEA level was more than 5 ng/ml, as listed in Table 1.

Target tumor cell lines were selected after confirmation of CEA expression on the cell surface. For the MKN45 and GT3TKB cell lines, the expression was apparent on the former but very slight on the latter. Since MKN45 and GT3TKB carry the same MHC class I molecules, HLA-A2402/B5201/Cw1202, a combination observed frequently in the Japanese (Tokyo Laboratory, Shionogi Biochemicals, personal communication), and CTL kill target tumor cells in a manner solely restricted to the type of MHC class I molecule,

Table 1 HLA typing of patients and their serum carcinoembryonic antigen (CEA) levels

Patient	Age (years)	Primary cancer	HLA-ABC typing	Serum CEA level (ng/ml)
1	47	Intrahepatic bile duct carcinoma	A24/B44/Cw1	5.6
2	74	Gastric carcinoma	A24/B35/Cw3,7	6.1
3	73	Duodenal papilla carcinoma	A24/B51,60/Cw3	6.6
4	62	Gastric carcinoma	A24/B76/Cw3,5	11.4
5	69	Biliary duct carcinoma	A24,33/B12/Cw1	17.5
6	48	Gastric carcinoma	A24/B50/Cw3	21
7	73	Gall bladder carcinoma	A2,24/B54,59/Cw3,7	24.6
8	34	Gastric carcinoma	A2,24/B35,61/Cw3	25

these are suitable naturally occurring target and control cell lines, respectively, for the detection of the activity of CEA-reactive CTL derived from patients carrying HLA-A2402.

Growth responses of the lymphocytes cultured on the autologous fixed adherent cells

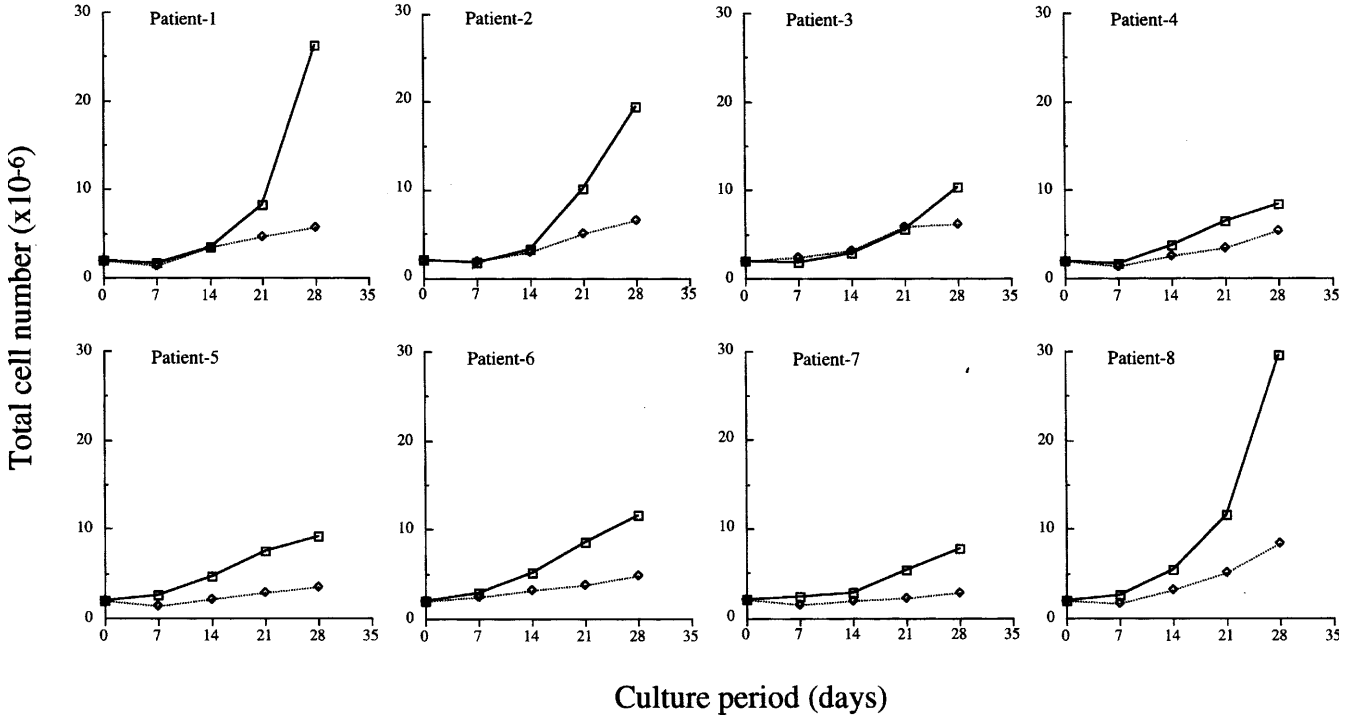
Figure 1 shows growth curves of the lymphocytes derived from eight patients tested in the present study. The peripheral-blood-derived adherent cells had been previously loaded with HLA-A24-restricted CEA peptide [CEA652(9)] and fixed with paraformaldehyde, then incubated with fresh live PBMC from the same patient, as described in Materials and methods. In a typical case, such as that of patient 1, the number of lymphocytes increased rapidly on the fixed cell layer previously loaded with the CEA peptide after 14 days. No essential increase was observed in the number of lymphocytes on the fixed cells without pre-loading of the CEA peptide. Similarly clear lymphocyte growth responses were seen in cells from patient 2 and patient 8, but those of other patients showed little growth response.

Cytotoxic activity of the cultured lymphocytes

In our laboratory, a non-radioisotopic crystal violet staining assay (Liu et al. 1995) has been used to determine the killing activity of killer lymphocytes on adhesive target tumor cells. Since this assay is safe, of low cost, and can be used for coculturing effectors and targets for longer than 6 h, it is as sensitive for assessment of the killing activity of the lymphocytes as the standard ^{51}Cr-release assay (Liu et al. 1995) or the europium-release assay (Volgmann et al. 1989). The absorbance at 570 nm of the target cells at the start of the killing assay was taken as 100%. Since the target cells grew during the 24-h incubation, the percentage of the surviving control target cells exceeded 100%, as shown in Fig. 2. If the surviving target cells did decrease to less than 100% after the 24-h incubation, the lymphocytes had clearly killed the target cells and not simply inhibited their growth without any killing. Therefore, we considered the CTL induction successful if the surviving target cells were reduced to less than 100% in the killing assay at an E/T ratio of 10 or less.

When the lymphocytes of patient 1 were tested for their cytotoxic activity on live CEA-producing MKN45 cells, killing was apparent only in the effectors induced on the CEA-peptide-pulsed fixed adherent cells (Fig. 2a). In contrast, the lymphocytes cultured on the fixed adherent cells without CEA peptide pre-loading did not generate the killing response to the target cells (Fig. 2b). The CTL seemed to kill the CEA652(9)-pulsed GT3TKB cells but not the native GT3TKB cells, although their killing activity at the E/T of 10 just failed to reduce the surviving target cells to less than 100% (Fig. 2a, Table 2). A higher E/T ratio was not tried because of the shortage of the effector lymphocytes. With another control of GT3TKB cells pulsed with

Fig. 1 Cumulative growth curves of the cultured lymphocytes from each patient. The cells were cultured with fixed adherent cells preloaded with CEA652(9) (TYALFVSNL) peptide (□) or without carcinoembryonic antigen (CEA) peptide (◇)



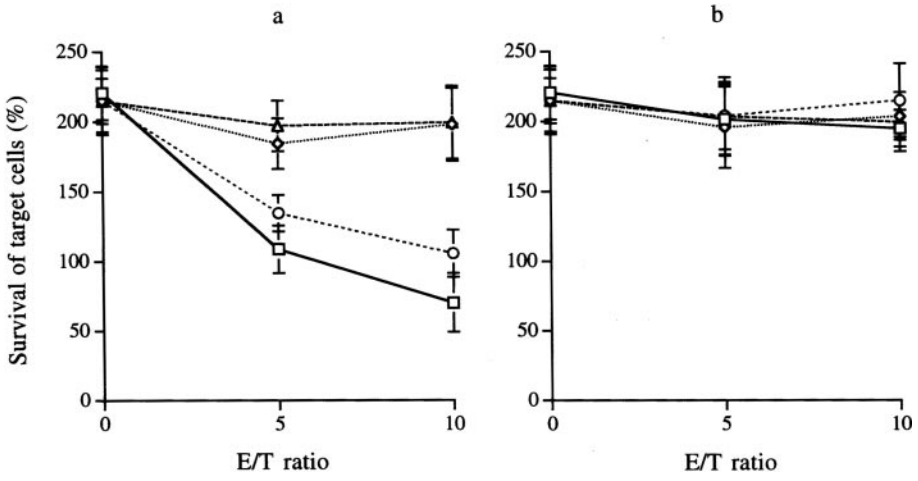


Fig. 2a, b Killing activity of cytotoxic T lymphocytes (CTL) from patient 1 induced on CEA652(9)-peptide-pulsed and fixed adherent cells from peripheral blood mononuclear cells (PBMC). The PBMC of patient 1 were stimulated on autologous fixed adherent cells preloaded with CEA652(9) peptide for 1 h (**a**) or without the peptide (**b**) for induction of the CTL. Live target cells were incubated in the presence of CEA652(9) peptide for 1 h and submitted to the killing assay. The crystal violet staining assay (see Materials and methods) was used to determine the killing activity of the CTL against CEA-producing MKN45 cells (□), CEA-non-producing GT3TKB cells (◇), GT3TKB cells pulsed with the CEA652(9) peptide (○), and GT3TKB cells pulsed with control irrelevant FLU38(10) peptide (△). The killing assay was performed for 24 h at the E/T ratios indicated. The A_{570} of each well of control target cells was separately measured at the start of the killing assay and this value was taken as 100% when calculating 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%. Control target cells were GT3TKB cells pulsed with no peptides or with an irrelevant control peptide FLU38(10). Each point represents the mean of four replicates accompanied by an error bar showing the SD

irrelevant peptide FLU38(10), the effectors did not show any killing activity (Fig. 2a).

Quite similar results were obtained with cells from patient 2, as summarized in Table 2. Patient 3 whose serum CEA level was about as high as that of patients 1 and 2 did not develop CTL, though his lymphocyte population reduced the surviving target cells to 107% at the E/T ratio of 10 (Table 2).

In contrast to these cases, the CTL derived from patient 8, whose serum CEA level was 25 ng/ml, killed CEA652(9)-pulsed GT3TKB but did not kill strongly CEA-producing MKN45 cells, although they reduced the surviving target cells from 196% at an E/T ratio of 0 (control) to 123% at an E/T ratio of 10 (Table 2). For all the other patients, the lymphocytes cultured for CTL induction showed little or no killing activity at an E/T ratio of 10 against the CEA-peptide-pulsed GT3TKB cells or MKN45 cells (Table 2).

For confirmation, the lymphocytes described above were then submitted to a standard europium-release assay (Volgmann et al. 1989) against natural-killer-cell-sensitive K562 cells and lymphokine-activated-killer-cell-sensitive Daudi cells that were tested at E/T ratios of 5 and 10. Both of the target lines were cultured in suspension and therefore the crystal violet staining assay was not suitable. However, no killing activity was observed with any of the lymphocytes tested (data not shown).

Table 2 Summary of activities of the effector lymphocytes against the target cell lines. The data show the percentage of surviving target cells remaining after the 24-h incubation in the killing assay. The A_{570} of each well of control target cells was separately measured at the start of the killing assay and this value was taken as 100% in when calculating 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%

Patient	E/T ratio	Surviving target cells (%)		
		MKN45	GT3TKB	
			With CEA peptide	Without CEA peptide
1	0	221	215	215
	5	109	134	185
	10	70	105	198
2	0	169	183	183
	5	128	120	170
	10	86	106	161
3	0	169	183	183
	5	155	127	155
	10	107	111	159
4	0	169	183	183
	5	151	145	165
	10	125	135	158
5	0	205	187	187
	5	173	146	165
	10	161	158	160
6	0	213	204	204
	5	182	181	188
	10	167	179	173
7	0	169	183	183
	5	148	169	179
	10	142	140	160
8	0	196	177	177
	5	140	109	131
	10	123	99	148

Table 3 Phenotype of the effector lymphocytes from each patient as revealed by flow cytometry

Patient	Phenotype (%)			
	$CD3^+$	$CD56^+$	$CD4^+$	$CD8^+$
1	89.4	2.6	12.4	78.3
2	93.6	0.2	14.6	81.2
3	92.5	0.3	36.8	56.4
4	76.3	10.9	16.6	43.4
5	62.6	1.6	38.6	22.8
6	82.7	11.8	81.2	6.6
7	66.5	1.1	30.1	26.8
8	94.6	1.6	18.6	72.8

Phenotype of the effector lymphocytes from patients

The phenotype of the effector lymphocytes cultured for CTL induction are listed in Table 3. For example, those of patient 1 consisted of 89.4% $CD3^+$ cells, 2.6% $CD56^+$ cells, 12.4% $CD4^+$ cells, and 78.3% $CD8^+$ cells. In contrast to the high proportion of $CD8^+$ cells in the CTL population, the control lymphocytes of patient 1 cultured on the fixed adherent cells, without CEA peptide pre-loading, consisted of 70.5% $CD3^+$ cells, 15.5% $CD56^+$ cells, 3.4% $CD4^+$ cells, and 18.8% $CD8^+$ cells. Although the phenotypes of the control lymphocytes were not determined for each patient, only for patient 1, the effector lymphocytes from patients 2 and 8 also revealed higher ratios of $CD8^+$ cells than those from patients 3, 4, 5, 6, and 7. $CD56^+$ cells, including natural killer cells, were less than 3% in the effector lymphocytes from patients 1, 2, and 8.

Inhibition of killing activity with antibodies

As shown in Fig. 3, inhibition of the killing of target MKN45 cells by CTL from patient 1 was observed when the lymphocytes were treated with monoclonal antibodies against CD3, CD8 and MHC class I molecules just before the crystal violet staining assay. In contrast, the killing was not blocked by anti-CD4 and anti-MHC-class II antibodies or blocking was so low that it was considered to be substantially non-specific.

Production of cytokines

Since CTL are known to produce cytokines including IFNγ and TNFα in an antigen-specific manner, we assayed these cytokines in the culture medium of CTL from patient 1 after stimulation with the autologous fixed adherent PBMC previously loaded with the CEA652(9) peptide. As shown in Fig. 4, the effector cells produced these cytokines but showed essentially no cytokine production when stimulated with the fixed adherent cells without CEA-peptide pre-loading.

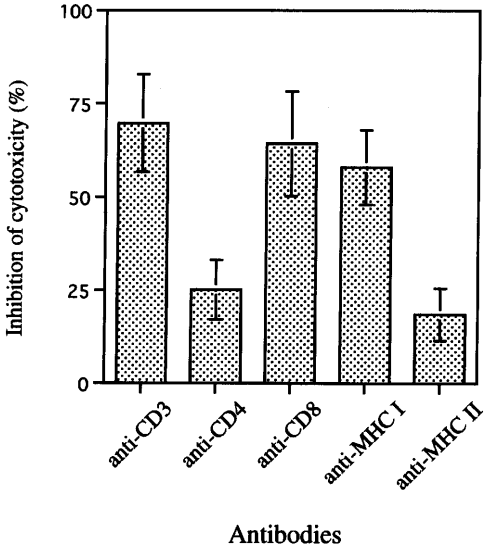


Fig. 3 Inhibition of cytotoxicity of CTL from patient 1 with monoclonal antibodies. Inhibition assays were carried out at an E/T ratio of 10. The effector CTL were pretreated with the indicated anti-CD monoclonal antibody at 4 °C for 4 h. Target MKN45 cells were pretreated with an antibody against MHC class I or MHC class II molecules at 37 °C for 4 h. The effector cells and the target cells were incubated for 6 h at 37 °C. Each point represents the mean of four replicates accompanied by an error bar showing the SD

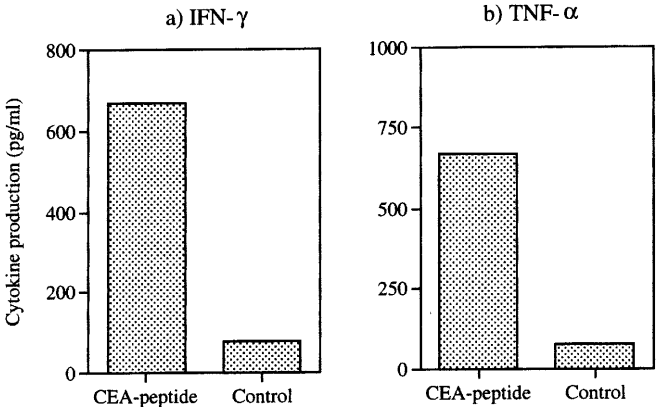


Fig. 4a, b Cytokine production by CTL from patient 1 after stimulation with autologous CEA652(9)-peptide-preloaded and fixed adherent cells. The lymphocytes were stimulated with autologous fixed adherent cells that had (*CEA-peptide*)or had not been pulsed (*Control*) with the CEA652(9) peptide. The 24-h culture supernatants were collected for the assay of interferon γ (IFNγ, **a**) and tumor necrosis factor α (TNFα, **b**) (see Materials and methods). Results are expressed in pg cytokine/ml culture supernatants

Discussion

The present results suggest that the lymphocyte population cultured on the autologous fixed adherent PBMC pre-loaded with an antigenic epitope peptide contain CTL responding to the antigen-producing carcinoma cells (Fig. 2) and that the CEA-reactive CTL are inducible in 38% of the cancer patients with elevated

serum CEA levels (Tables 1, 2). Also, in a typical case, increased release of IFNγ and TNFα was observed from the lymphocytes stimulated with the autologous fixed adherent cells previously pulsed with the antigenic peptide (Fig. 4), and this was consistent with the CTL activity (Fig. 2a). Although CTL from patients 1, 2, and 3 produced slightly unbalanced killing (Table 2) of MKN45 and GT3TKB, both of which carry the same MHC class I alleles, these CTL are predicted to kill the autologous CEA-producing cancer cells that, to our regret, have not been cultured in vitro. We could not detect significant killing activities in the present lymphocyte population against K562 cells and Daudi cells, suggesting that, as relatively low percentages of $CD56^+$ cells were observed in the effector lymphocytes (Table 3), the induced effector lymphocytes contained few or no natural killer cells or lymphokine-activated killer cells. A similar phenomenon has been described in a previous report (Kim et al. 1998).

However, there were differences in the efficacy of induction of CTL and in the cytotoxicity of induced effector lymphocytes among the eight patients recruited (Table 2). At present, we are not able to provide evidence to explain the difference, but it appears at least to reflect the growth responses of the PBMC to the autologous fixed adherent PBMC pre-loaded with CEA652(9) peptide (Fig. 1), which corresponded well to the successful induction of CTL (Table 2). After 2 weeks in the CTL induction culture (therefore after the second re-stimulation with fixed adherent cells pre-loaded with the antigenic peptide), PBMC from patients 1, 2, and 8 exhibited rapid growth of lymphocytes. The majority of these lymphocytes were $CD8^+$ T cells (Table 3), which must contain the typical MHC-class-I-restricted $CD3^+CD4^-CD8^+$ CTL described by many investigators. This is suggested by the results shown in Fig. 3, in which monoclonal antibodies against CD3, CD8, and MHC class I, but not those against CD4, strongly inhibited cytotoxicity of the effector lymphocytes. MHC class II molecules were not involved in the cytotoxic response of the CTL.

Since many other tumor antigenic peptides have been identified as being recognized by HLA-A2 and/or HLA-A24-restricted CTL, such as HER-2/neu (Fisk et al. 1995; Peoples et al. 1995), MART-1 (Kawakami et al. 1994), MAGE-1 (Traversari et al. 1992), MAGE-3 (Tanaka et al. 1997), MUC-1 (Takahashi et al. 1994; Agrawal et al. 1996), tyrosinase (Kang et al. 1995) and gp100 (Kawakami et al. 1995), the present method for the induction of the corresponding CTL will also apply to these peptides and will be eligible for use in adoptive immunotherapy of tumor-bearing patients. However, we do not consider that the present CEA-reactive CTL are directly useful for treating cancer patients. As has been discussed by Nukaya et al. (1999), the sequence of the antigenic peptide CEA652(9), TYACFVSNL, is identical to that of its non-specific cross-reacting antigen counterpart and, therefore, there is a finite probability of cross-reaction to the non-specific cross-reacting antigen, which is present in a variety of normal tissues (Thompson et al. 1991). Since, for ethical reasons, we were reluctant to obtain fresh PBMC repeatedly from the suffering carcinoma patients, we have not tested quantitatively whether the present CTL could kill autologous normal leukocytes.

It is well known that dendritic cells can be developed from PBMC cultured for a week with granulocyte/macrophage-colony-stimulating factor and IL-4 (Sallusto and Lanzavecchia 1994; Romani et al. 1994). Dendritic cells are the ''professional'' antigen-presenting cells that can strongly stimulate naive resting T cells and initiate a primary T cell response when pulsed with antigenic peptide or protein (Inaba et al. 1990; Mehta et al. 1994; Bakker et al. 1995). However, dendritic cells are sparse in PBMC and do not proliferate readily in vitro in spite of the report of Romani (1994). Therefore heavy leukapheresis is sometimes required for dendritic cell preparation in cancer patients, who quite often have damaged bone marrow (the source of the cells) because of treatments with radiation and/or antitumor drugs.

In contrast, the present technique to prepare the adhesive PBMC is very simple and rapid (see Materials and methods). As previously reported (Kim et al. 1998), CEA protein bound to latex beads can be processed for presentation of the various antigenic peptides by MHC class I molecules in the adherent PBMC and can generate a CTL response in vitro from fresh PBMC cultured on the fixed adherent PBMC. This, together with the present results, indicates that fixed adherent PBMC are useful antigen-presenting cells. They can also be kept in a refrigerator until use, which considerably aids handling and the process of antigen presentation to stimulate autologous PBMC ex vivo. The present method for CTL induction will be an improvement on the method in which dendritic cells have to be precultured and pulsed with the antigenic peptides every time for the induction and re-stimulation of autologous CTL (Bakker et al. 1995).

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