CHAPTER II

Induction of autologous cytotoxic T lymphocytes with a CEA-derived HLA-A2402-binding peptide from peripheral blood mononuclear cells of CEA-positive cancer patients

Summary

Inducibility of carcinoembryonic antigen (CEA)-specific and HLA-A24 restricted autologous cytotoxic T lymphocytes (CTL) was tested in patients carrying moderately high (5 - 10 ng/ml) and high (> 10 ng/ml) serum CEA levels. By culturing patient's fresh peripheral blood mononuclear cells (PBMC) on formalin-fixed autologous adhesive PBMC that have been pre-loaded with CEA652(9), a tumor antigenic peptide derived from CEA, 2 out of 3 moderately high-CEA patients and 1 out of 5 high-CEA patients developed autologous CTL. Two of them killed CEA-protein-producing gastric cancer cells carrying HLA-A2402 and the other one killed CEA-non-producing stomach cancer cells pre-loaded with CEA652(9). Specificity of the CTL was confirmed in a patient through the killing of CEA-non-producer with or without the antigenic peptide pre-loading. These results suggest that, although breaking of the tolerance or anergy to HLA-type-matched

CEA-producing cells may be difficult in the cancer patients who are carrying high serum CEA levels, the use of a single CEA-derived antigenic peptide will be eligible for use in the *ex vivo* induction of CEA-specific CTL without tedious pre-culture of the antigen-presenting dendritic cells.

Introduction

The identification and selection of tumor-associated antigens (TAA) for the development of T-cell-based immunotherapy for cancer are presently a high priority activity of various research groups [129-131]. Because cytotoxic T lymphocytes (CTL) are likely the most effective element for anti-tumor immune responses, much emphasis has been placed on the definition of the TAA-derived peptide epitopes for these effector cells. 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, 9-10 amino acids long, that are complexd to MHC class I molecules expressed on the cell surface [20,92]. CTL epitope from numerous TAA have identified by several methods [132-134]. Tumor antigen-specifc CTL have been induced from peripheral blood mononuclear cells (PBMC) isolated from normal individual using professional antigen-presenting cells (APC) such as dendritic cells (DC), which were pulsed with candidate peptides derived from TAA sequences. These peptides were selected first by the presence of major histocompatibility complex (MHC) binding motifs and theresfter by their capacity to bind with relatively high affinity to purified MHC molecules [135]. Recently, the

identification of tumor-specific and tumor-associated antigen recognized by human cytotoxic T lymphocytes is being studied mainly for potential immunotherapeutic benefit in the treatment of cancer. Several studies have also reported that CTL can be induced against defined epitope peptides of a range of human tumor-associated antigens (TAA), such as HER-2/neu [129,136], MART [130], MAGE-1 [137], MAGE-3 [131], CEA [144], MUC-1 [145] and tyrosinase [128].

Carcinoembryonic antigen (CEA) is a 180kDa glycoprotein that is extensively expressed in the vast majority of colorectal, gastric and pancreatic carcinomas, approximately 50% of breast cancers and 70% of non-small-cell lung cancers [121]. CEA is also present, but usually at lower concentrations, in the normal colon epithelium and in some fetal tissues [121]. Previous studies have shown that human monocytes-derived dendritic cells (DC) pulsed with CEA-derived peptide or 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 (without record of the CEA level) [143] or healthy donors [146-149]. These CTL showed an antigen-specific and HLA-A2-, A-24- or A3-restricted lysis of target cells pulsed with the antigenic peptide and established tumor cell line expressing CEA. We have reported recently that HLA-A24-restricted and CEA-specific CTL were easily generated by

culturing healthy human PBMC on formalin-fixed autologous adhesive PBMC which had been loaded with CEA protein bound to latex beads [150]. The CTL could kill CEA-producing carcinoma cells and efficiently recognized the CEA epitope peptide TYACFVSNL [CEA652(9)]. We also showed induction of autologous CTL on the healthy donor adherent cells pulsed with CEA652(9) peptide *in vitro*. On the basis of these results, we have tried to induce HLA-A24-restricted and CEA-specific autologous CTL from PBMC of carcinoma patients showing high serum CEA levels who may have undergone tough tolerance or anergy to CEA-produuding cells. Induction of such CTL was successful for 3 out of 8 patients examined.

Material and methods

Target cells and culture condition

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 [124] and, as a control, a stomach adenocarcinoma cell line GT3TKB that is a non-producer of CEA (see Fig. 6a and 6b, Chapter I). The HLA phenotype was determined by the method of Blasczyk et al [125]. These cell lines were cultured according to the method described in *Material and methods*, Chapter I.

Peptides and reagents

The HLA-A24-rerstricted CEA peptide (amino acids 652-660, TYACFVSNL), a control irrelevant peptide (amino acids 38-47, REYIQMCTEL) and various reagents are shown in *Material and methods*, Chapter I.

Preparation of antigen-presenting cells and pulsing with CEA peptide

Human PBMC were prepared by the conventional Ficoll-Paque centrifugation method from heparinized peripheral blood of each

patient bearing a CEA-expressing carcinoma who has been identified, first by serological method and then by genotyping, as carrying the HLA-A2402 subtype. The cells were washed once with PBS, then once with RHAMa medium supplemented with 5% autologous plasma and centrifuged at 1,400 rpm for 10 min at room temperature as previously described [150].

The separated PBMC ($2x10^6$ cells) were allowed to adhere to a well of 24-well culture plates for 2 hrs at 37 °C in RPMI 1640 medium containing 10% FBS. The adherent cells, which had monocyte/macrophage-like morphology, were further cultured in the 24-well plate with 2 ml of the medium containing CEA peptide (50 μg). After 2 hr in the culture, the adherent cells were fixed with 3% paraformaldehyde in PBS for 1 hr at room temperature and washed thoroughly with the culture medium before the induction of CTL (as described in *Material and methods*, Chapter I).

Induction of antigen-specific CTL from PBMC

On the fixed adherent cells, freshly prepared PBMC ($1x10^6$ cells) from the same carcinoma patient were added with 2 ml of RHAMa medium supplemented with human interleukin(IL)-1b (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 with half medium change every other day. The effector cells were re-stimulated weekly to a total of 3 re-stimulations at an effector/adherent cell ratio of 2 on the fixed adherent cells which were renewed at every re-stimulation.(see Fig. 13).

Cytotoxicity assay

The assay for cell-mediated killing of target cancer cells was performed *in vitro* using the crystal violet (CV) staining method as described in *Material and methods*, Chapter I.

To detect activities of natural killer cells or lymphokine-activated killer cells, a standard europium release assay was performed in the 4-hr coculture of the effector lymphocytes and the target K562 cells and Daudi cells as described [151]. Briefly, A total of $1x10^6$ target cells were labeled with europium diethylenetriamine pentaacetate (Eu^{3+}) for 20 min at 4 °C. After washes 5-6 times with complete medium, 10^6 europium-labeled target cells and serial dilutions of effector cells at E/T ratio of 5:1 and 10:1 were incubated in 200 µl of RPMI 1640 with 10% heat-inactivated FBS in 96-well U-bottom plates. The plates were centrifuged at 1400 rpm for 5 min and incubated at 37 °C and 5% $CO_2$for 4 hr. After incubation the cells were centifuged and 20 µl of the supernatants were transferred to the cups of flat-bottomed microtiter

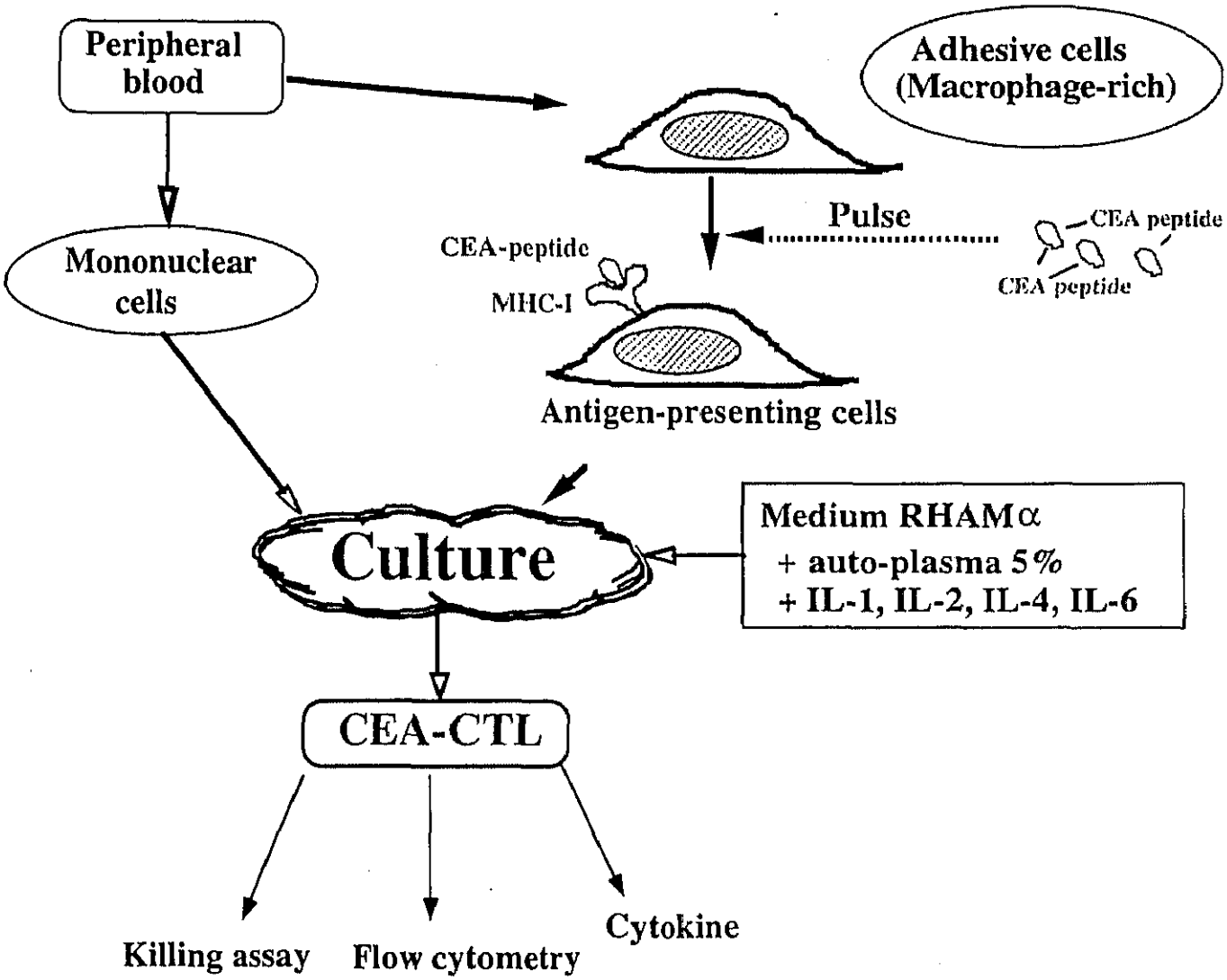

Fig. 13. Induction of Autologous CEA-CTL

strips containing 200 µl of an enhancement solution. After thorough mixing for 5 min at room temperature fluorescence was measured in a time-resolved fluorometer. Spontaneous release of target cells was less than 25%. Each assay was performed four replicated and percentage of surviving target cells was calculated according to following formula:

Surviving target cells (%) = [(experimental release-spotaneous release)] / [(maximum release-spotaneous release)] x 100

The maximum release was the total release obtained from target cells treated with 0.5% Triton X-100, and spotaneous release was the normal release of Eu chelate during test time without effector cells.

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 in *Material and methods*, Chapter I. Briefly, these antibodies were used at a final concentration of 20 µg/ml. Target MKN45 cells were precultured overnight in 96-well plates at $1x10^4$ cells/well. These target cells were pretreated also as described in *Material and methods*, Chapter I. 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 hr. The adherent target cells were quantified by the

CV-staining described in *Material and methods*, Chapter I.

The inhibition of cytotoxic activity was calculated according to following formula:

% inhibition = (A-B)/(T-B) x 100

where A is A570 value of the target cells to which the effector cells were added, in which the target cells or the effector cells were pretreated with a monoclonal antibody; B is A570 value of the target cells to which the effector cells were added and neither cell type was pretreated with any monoclonal antibodies; and T is A570 value of the target cells to which neither CTL nor antibodies were added.

HLA Typing

The HLA phenotyping of each patient was performed using Terasaki oriental HLA-ABC well Tray (One Lambda, Inc, Canoga Park, CA).

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

For determination of IFNg and TNFa production (as described in Materiala and Methods, Chapter I), supernatants of the effector cells exposed for 24 hr to peptide-pulsed APC at a responder:stimulater ratio of 2 were submitted to ELISA according to the manufacturer's protocol (phaMinger, San Diego. CA). The results were expressed in pg/ml.

Results

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

Patients expressing HLA-A2402 on their cell surface were subjected to the present study, since approximately 60% of Japanese carry HLA-A24 gene and more than 98% of HLA-A24 alleles are known to be HLA-A2402 (Tokyo Laboratory, Shionogi Biochemicals, personal communication). This high percentage of 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 5ng/ml at the Univesity of Tsukuba Hospital, patients for the present study were therefore selected whose serum CEA level was more than 5 ng/ml as listed in Table 6. We arbitrarily subdivided the patients into two groups, i. e., moderately high CEA group with 5.1 -10.0 ng/ml and high CEA group with more than 10.0 ng/ml.

Target tumor cell lines were selected after confirmation of CEA expression on the cell surface. For 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, of which combination was observed

frequently in Japanese (Tokyo Laboratory, Shionogi Biochemicals, personal communication), and CTL kill target tumor cells in the manner solely restricted to the type of MHC-class I molecules, these are suitable naturally occurring target and control cell lines, respectively, for the detection of the CEA-specific CTL activity that are derived from patients carrying HLA-A2402.

Growth responses of the lymphocytes cultured on the fixed adherent cells derived from the patients

Fig. 14 shows growth curves of the lymphocytes derived from 8 patients tested in the present study. The peripheral blood-derived adherent cells containing macrophages have been previously loaded with HLA-A24-restricted CEA peptide (CEA652(9)) and fixed, then incubated with live autologous PBMC as described in Materials and Methods. On a typical case such as 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 growth responses of the lymphocytes were seen in Patient-2 and Patient-8, but those of other patients showed little growth responses.

Cytotoxic activity of the cultured lymphocytes

In our laboratory, a non-radioisotopic CV staining assay [65] has been used for determination of killing activty of killer lymphocytes on adhesive target tumor cells. Since this CV assay is safe, of low cost, and is amenable for coculturing effectors and targets for longer than 6 hr, it is as sensitive for assessment of the killing activity of the lymphocytes as the standard ^{51}Cr-release or europium-release assays [65,151]. 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-hr incubation, the percentage of the surviving control target cells exceeded 100% as shown in Fig. 15a and b. If the surviving target cells did decrease to less than 100% after the 24-hr incubation, the lymphocytes have clearly killed the target cells and not simply inhibited the growth of the target cells without any killing. Therefore we defined that CTL induction was 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, apparent killing was observed only in the effectors induced on the CEA-peptide-pulsed fixed adherent cells (Fig. 15a). In contrast, the lymphocytes cultured on the fixed adherent cells without CEA-peptide pre-loading did not generate the killing response against the target cells (Fig. 15b).

The CTL seemed to have killed the CEA652(9)-pulsed GT3TKB cells but not the native GT3TKB cells, although their killing activity at the E/T of 10 was slightly short of reducing the surviving target cells to less than 100% (Fig. 15a, Table 7). A higher E/T ratio was not tried because of the shortage of the effector lymphocytes. To another control of GT3TKB cells pulsed with irrelevant peptide FLU38(10), the effectors did not show any apparent killing activity (Fig. 15a).

Quite similar results were obtained in Patient-2 as summarized in Table II. Both Patient-1 and Patient-2 belonged to the moderately high CEA group (Table 6). However, Patient-3 whose serum CEA level was also moderately high (6.6 ng/ml) did not develop CTL, though his lymphocyte population reduced the surviving target cells to 107% at the E/T ratio of 10 (Table 7).

Contrary 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 apparently CEA-producing MKN45 cells (Table III). For all the other patients, who belonged to the high CEA group, 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 7).

For confirmation, the above described lymphocytes were then submitted to a standard europium release assay [151] 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 targets were cultured in suspension and therefore CV staining assay was not suitable. However, no killing activity was observed with any of the lymphocytes tested (data not shown).

Phenotype of the CTL from Patient-1

The phenotype of the Patient-1 CTL consisted of $CD3^+$ cells, 89.4%; $CD4^+$ cells, 12.4%; $CD8^+$ cells, 78.3%. Contrary to the high $CD8^+$ proportion in the CTL population, the control lymphocytes cultured on the fixed adherent cells without CEA-peptide pre-loading consisted of $CD3^+$ cells,70.5%; $CD4^+$ cells, 3.4%; $CD8^+$ cells, 18.8% [Table 8].

As shown in Fig. 16, inhibition of the killing activity of the CTL on the target MKN45 cells was observed when the lymphocytes were treated with monoclonal antibodies against CD3, CD8 and MHC-class I molecules just before the CV staining assay. In contrast, the killing was not, or to the low extent that was considered to be substantially non-specific, blocked by anti-CD4 and anti-MHC-class II antibodies.

Production of cytokines

Since CTL are known to produce cytokines including interferon(IFN)-γ and tumor necrosis factor(TNF)-α in an

antigen-specific manner, we assayed these cytokines in the culture medium of Patient-1 CTL after stimulation with the autologous fixed adherent PBMC previously loaded with the CEA652(9) peptide.

As shown in Fig. 17, the effector cells produced these cytokines but showed essentially no cytokine production when stimulated with the fixed adherent cells without CEA-peptide pre-loading.

Table 6. The HLA phenotyping of each patients and its seum CEA level

Patient	Primary cancer	HLA-ABC typing	Serum CEA level (ng/ml)
Moderately high CEA group (5-10ng/ml)			
1	Intrahepatic bile duct carcinoma	A24/B44/Cw1	5.6
2	Gastric carcinoma	A24/B35/Cw3,7	6.1
3	Duodenal papilla carcinoma	A24/B51,60/Cw3	6.6
High CEA group (>11ng/ml)			
4	Gastric carcinoma	A24/B76/Cw3,5	11.4
5	Biliary duct carcinoma	A24,33/B12/Cw1	17.5
6	Gastric carcinoma	A24/B50/Cw3	21
7	Gallbladder carcinoma	A2,24/B54,59/Cw3,7	24.6
8	Gastric carcinoma	A2,24/B35,61/Cw3	25

Since the threshold level of serum CEA has been set at 5 ng/ml at the University of Tsukuba Hospital, patients for the present study were therfore selected whose serum CEA level was more than 5 ng/ml. We arbitrarily subdivided the patients into two groups, i. e., moderately high CEA group with 5 - 10 ng/ml more than 10 ng/ml.

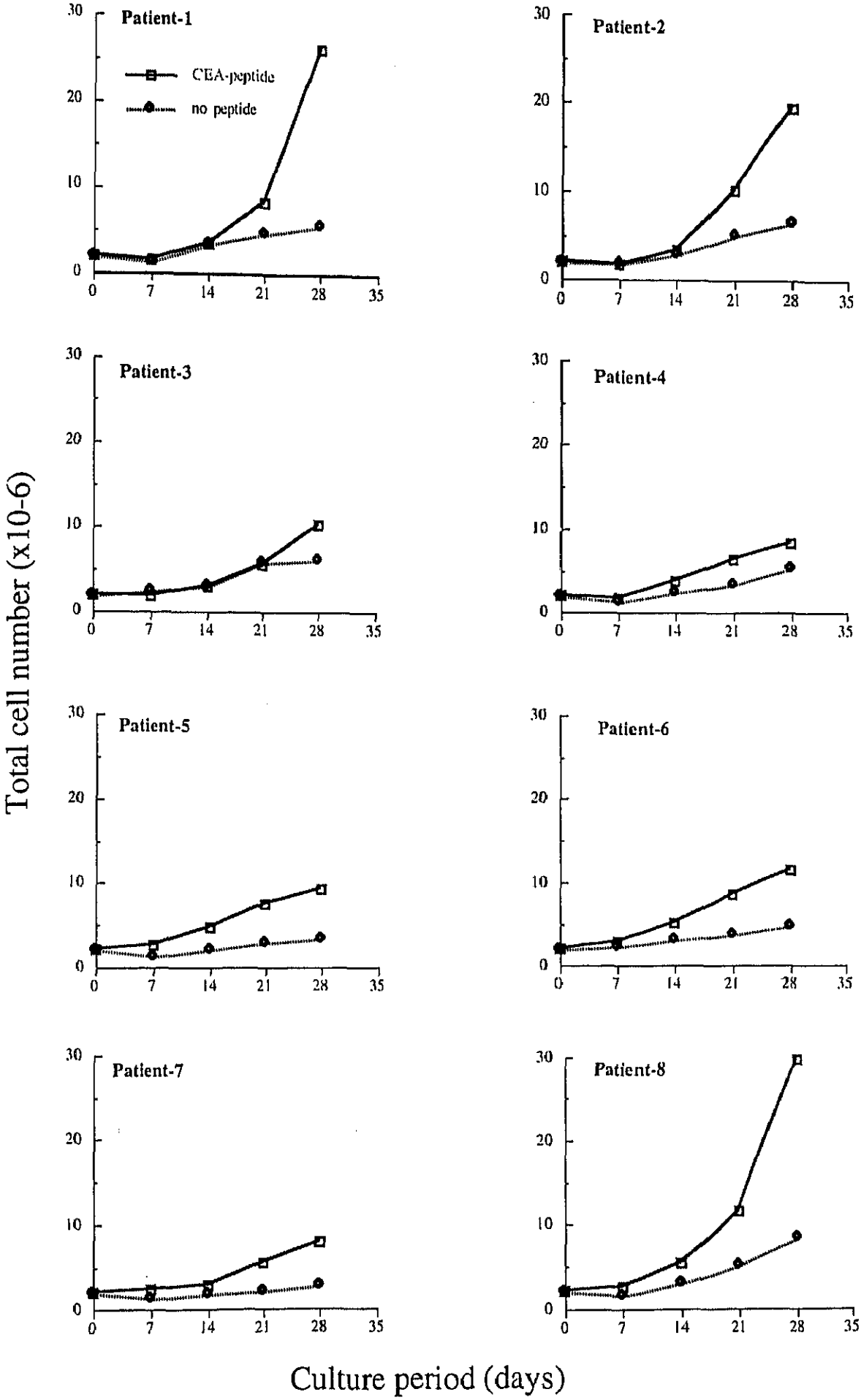

Fig. 14. Cumulative growth curves of the cultured lymphocytes in each patient.
The cells were cultured with fixed adherent cells pre-loaded with CEA peptide (□) or without CEA peptide (◇).

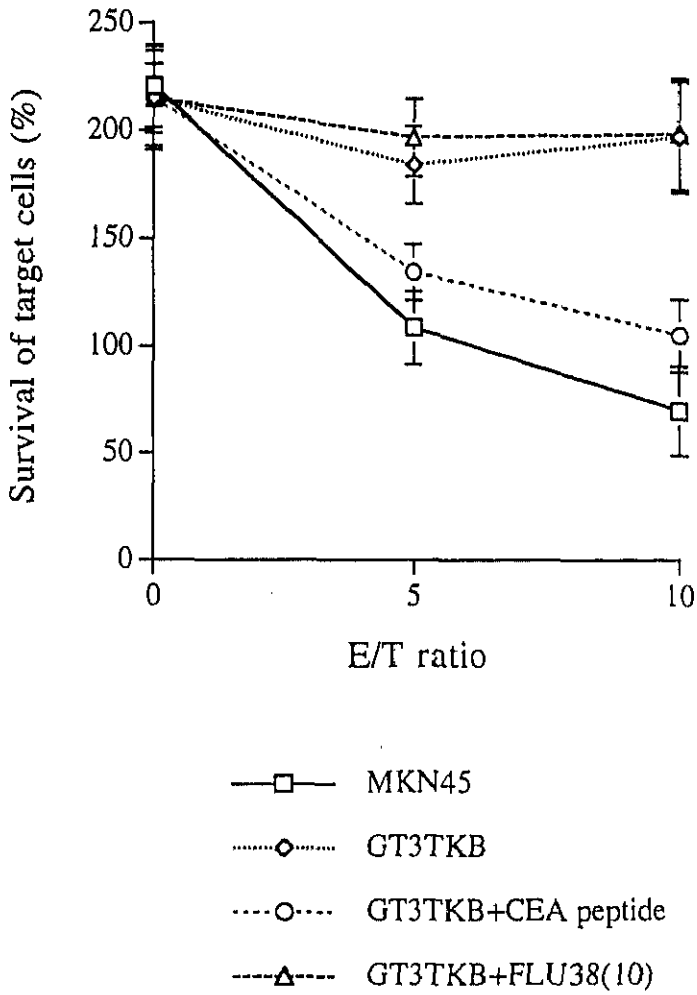

Fig.15a. Killing activity of Patient-1 CTL induced on CEA652(9) peptide-pulsed and fixed adherent cells from PBMC

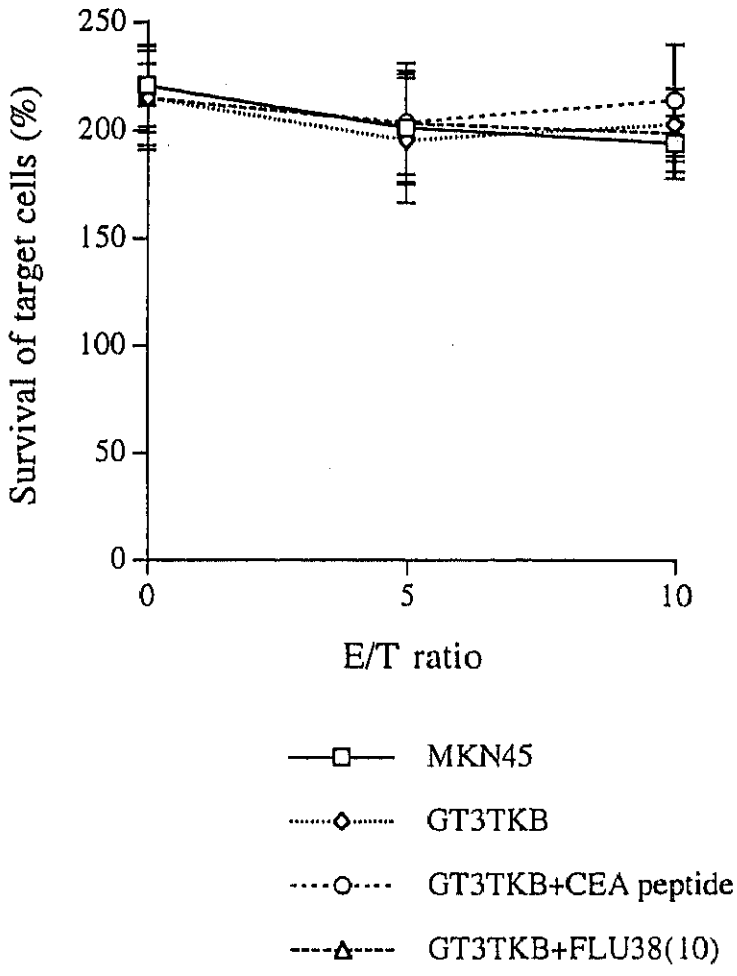

Fig.15b. Killing activity of Patient-1 CTL induced on fixed adherent cells without CEA652(9) peptide-pulsed

Table 7. Cytotxicity assay from each patient

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

Table 8. Surface phenotype analysis of CTL from Patient-1

Effector cells cultured with	Percentage positive cells		
	CD3	CD4	CD8
CEA-peptide	89.4	12.4	78.3
No peptide	70.5	3.4	18.8

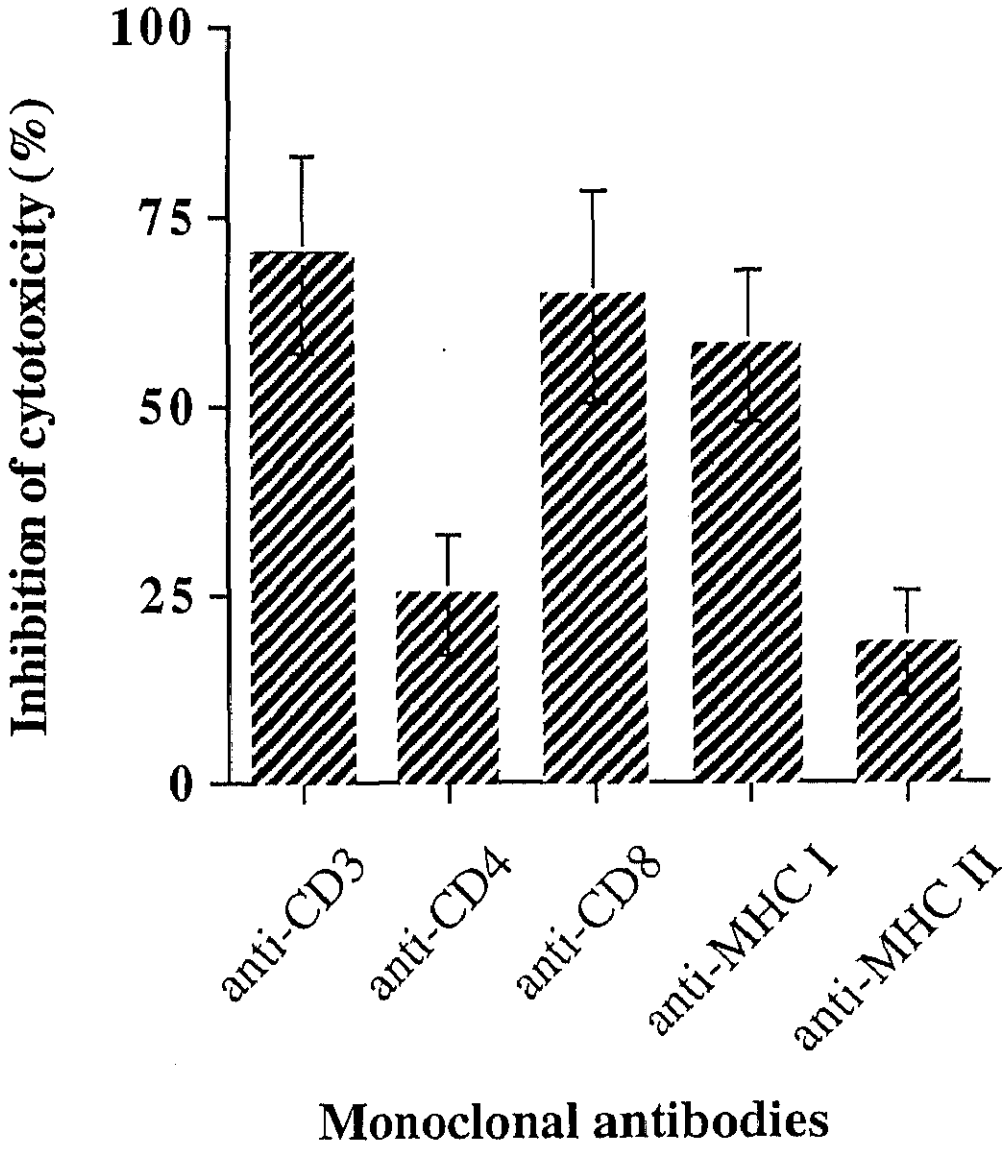

Fig. 16. Inhibition of cytotoxicity of Patient-1 CTL with monoclonal antibodies.
Inhibition assays were carried out at an E/T ratio of 10.

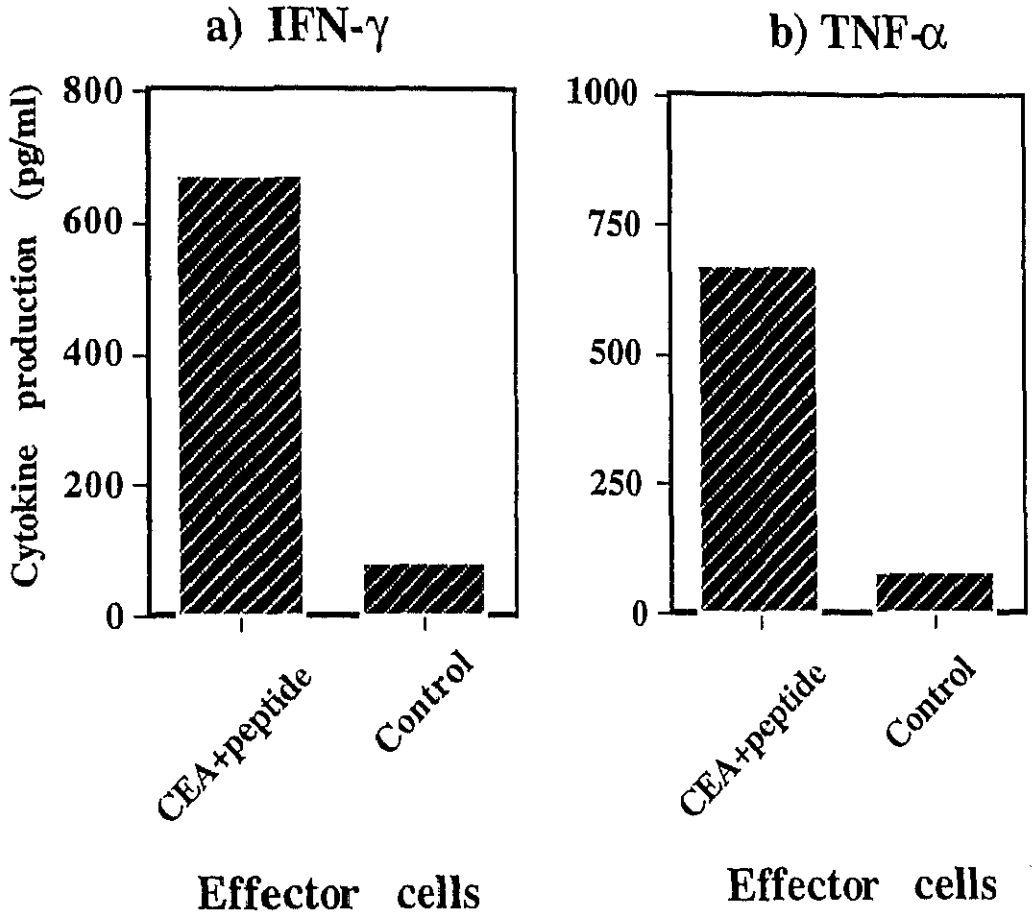

Fig. 17. Cytokine production from Patient-1 CTL after stimulation with autologous CEA652(9) peptide-loaded and fixed adherent cells.

Discussion

The present results suggest that the lymphocyte population cultured on the autologous fixed adherent PBMC pre-loaded with CEA652(9) peptide contain HLA-A24-restricted $CD8^+$ CTL specific to CEA-producing carcinoma cells (Fig. 15a and b and Fig. 16) and that the CEA-specific CTL are inducible in most of the cancer patients with moderately high serum CEA levels but in only 20% of the patients with high serum CEA levels (Table 6 and Table 7). Growth responses of the PBMC to the autologous adherent PBMC pre-loaded with CEA652(9) peptide (Fig. 14) corresponded well to the successful induction of CTL (Table 7). As confirmed in Fig. 16, MHC- class II molecules were not involved in the cytotoxic response of the CTL. Also, though in a typical case, increased release of IFN-γ and TNF-α was observed from the lymphocytes stimulated with the autologous fixed adherent cells pulsed with CEA652(9) peptide (Fig. 17) and this was consistent with the CTL activity (Fig. 15a). Although slightly unbalanced killings were observed in the patients-1, -2, and -8 against MKN45 and GT3TKB both of which carry the same MHC-class I alleles (Table 7), their CTL were 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 the induced lymphocytes contained few or no natural killer cells and lymphokine-activated killer cells. A similar phenomenon has been observed in a previous report [150]. Since many other tumor antigenic peptides have been identified for HLA-A2 and/or HLA-A24 such as CEA-derived peptides [146,148,150], MAGE-3 [131,155], MART-1 [157], gp100 [158] and tyrosinase [128], the present method will also be applicable to these peptides for the induction of corresponding CTL and will be eligible for use in adoptive immunotherapy of tumor-bearing patitents. However, we do not consider that the present CEA-reactive CTL are straightly useful for treatment of the cancer patients. As has been discussed by Nukaya et al. (146), the sequence of the antigenic peptide CEA652(9), TYACFVSNL, is identical to the non-specific cross-reacting antigen (NCA) counterpart and, therefore, the probability of cross reaction to NCA which is present in a variety of normal tissues (121) will be high. Since we ethically hesitated to obtain fresh PBMC repeatedly from the suffering carcinoma patients, we have not tested quantitatively that the present CTL may possibly kill autologous normal leukocytes.

We have recently reported that latex beads-bound CEA protein can be processed for presentation of the plural antigenic peptides by

MHC-class I molecules in the adherent PBMC and can generate a CTL response *in vitro* [150]. Therefore, together with the present results, it appears that adherent PBMC are useful as the antigen-presenting cells (APC) without having to purify or preculture dendritic cells (DC, the professional APC) that can strongly stimulate naive resting T cells and initiate primary T cell response when pulsed with antigenic peptide or protein [72,140,152]. It has been well known that DC can be developed from PBMC cultured for a week with GM-CSF and IL-4 [153,154]. However, DC are sparse in PBMC and are not proliferative *in vitro*. Therefore it sometimes requires heavy leukapheresis for DC preparation in the cancer patients who are quite often bearing damaged bone marrow (the source of DC) because of treatments with radiation and/or anti-tumor drugs.

In contrast, the present technique to prepare the adhesive PBMC is very simple and rapid (see Materials and Methods). As previously reported (150), latex beads-bound CEA protein can be processed for presentation of the plural 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. Therefore, together with the present results, it appears that fixed adherent PBMC are useful as the antigen-presenting cells, and they can be preserved in a refrigerator until use. This brought us great ease of handling and

practice of the antigen presentation to stimulate autologous PBMC ex vivo. The present method for CTL induction will be advantageous relative to the method in which DC have to be precultured and pulsed with the antigenic peptides every time for the induction and re-stimulation of autologous CTL (72).