CHAPTER I

In vitro induction of HLA-A2402-restricted and carcinoembryonic- antigen specific cytotoxic T lymphocytes on fixed autologous peripheral blood cells

Summary

HLA-A2402 restricted and carcinoembryonic antigen (CEA)-specific cytotoxic T lymphocytes (CTLs) were induced by culturing human peripheral blood mononuclear cells (PBMC) on formalin-fixed autologous adhesive PBMC that have been loaded with CEA-bound latex-beads. The CTLs killed the CEA-producing HLA-type matched cancer cells, but not the non producers of CEA, at an effector/target ratio of 10 within 24 hours. On the basis of available HLA-A24 binding peptides, we have also attempted to identify the epitope peptide recognized by the CTLs. The peptide CEA652(9), TYACFVSNL, stimulated the most strongly the CTLs when pulsed on HLA-A2402 expressing target cells. Other 9 peptides so far tested were also active, but less efficiently, to the CTLs. The CTLs failed to kill target cells pulsed with HLA-A2 binding CEA peptide, CAP-1. The CTLs were

also generated on the fixed adherent cells previously pulsed with the peptide CEA652(9). Cytotoxic activity of the CTLs was inhibited by monoclonal antibodies against CD3, CD8, and MHC-class I molecules. These results suggest that human autologous CTLs will be inducible on the autologous fixed PBMC without use of the cultured target cancer cells if tumor antigenic protein is available.

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 histocompatability complex (MHC) [81-83]. The existence of separate processing pathways for presentation of exogenous antigens provide a suitable model for understanding how major histocompatability complex (MHC) class II-restricted $CD4^+$ helper T-cell responses are generated against extracellular antigens while MHC class I-restricted $CD8^+$ cytotoxic T-cell responses are directed against cytosolic antigen [19,84]. These MHC-restricted T cell immune responses are depicted in Fig. 1.

Exogenous antigens are internalized by antigen-presentation cells (APCs), degraded in vesicular intracellular compartments, and loaded on MHC class II molecules in a post-Golgi compartment. In contrast, peptides derived from cytosolic antigens by the action of proteosomes are transported into the endoplasmic reticulum (ER) lumen by an adenosine triphosphate-dependent transporter associated with antigen presentation (TAP). In the ER lumen, a chaperone-mediated assembly generates a stable complex containing MHC class I heavy chain, β_2-microglobulin, and an antigenic peptide. This complex trafficks to the

cell surface, where it can be recognized by $CD8^+$ T cells [85,86]. These pathway are depicted in Fig. 2a and b.

Knowledge of the molecular details of the peptide-class I interaction derives from a large body of recent data, among which are the solution of the three-dimensional structure of the class I molecules and the resolution of the structure of the peptide binding groove occupied by naturally processed peptides or single peptide epitopes [87-91]. Furthermore, sequence analysis of naturally processed peptides bound to class I either as mixture or as individual peptides revealed unique features of class I ligands [20,92-94]. MHC class I-bound peptides are restricted in length, generally of 8 to 10 amino acid, and bear key amino acid at defined position. These features are referred to as peptide motifs [20,92].

This clear-cut dichotomy between an exogenous processing pathway for MHC class II restricted T cell response and an endogenous pathway for MHC class I restricted T cell response is supported by extensive experimental data. Evidence for the latter was provided by the demonstration that exogenous proteins or killed pathogens failed to stimulate MHC class I- restricted CTLs in vitro [95].

Generally, CTL induction usually follows processing of antigens via endogenous pathways before presentation on the cell surface in association with MHC class I molecules. Because of this special

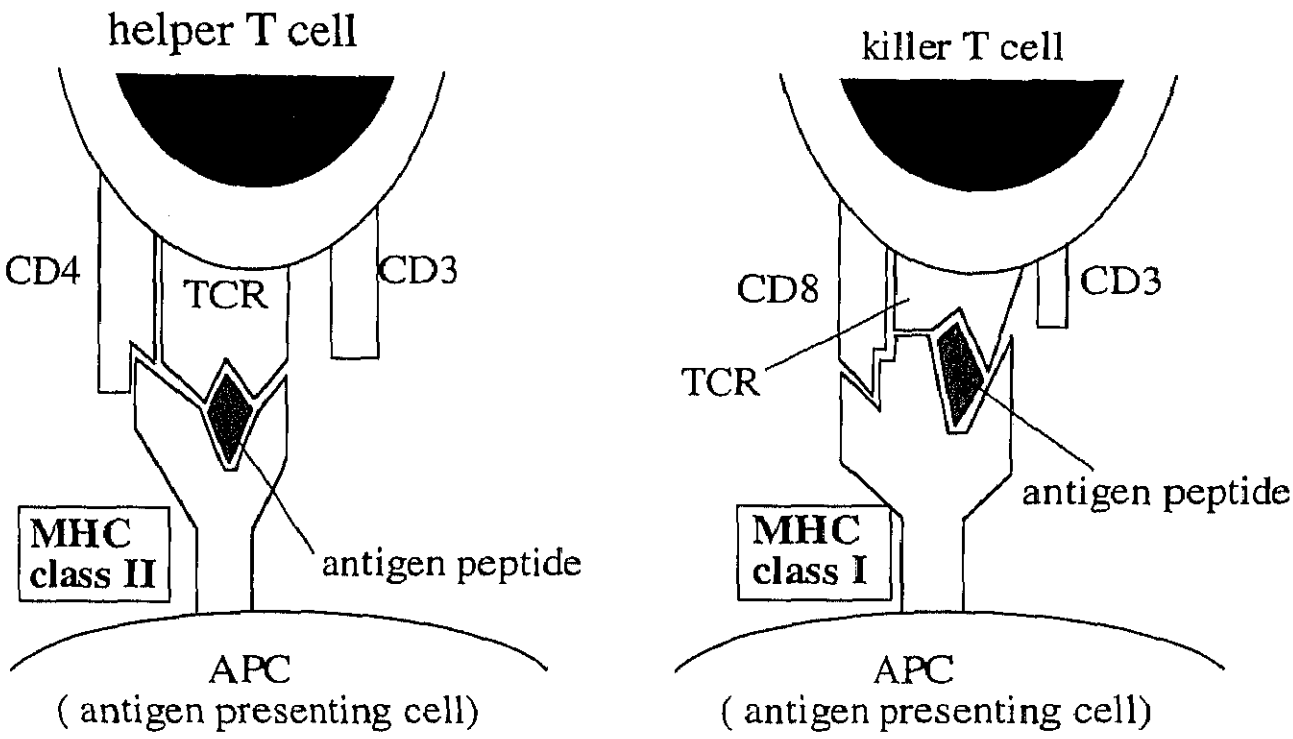


Fig. 1. MHC-restricted T cell immune responses

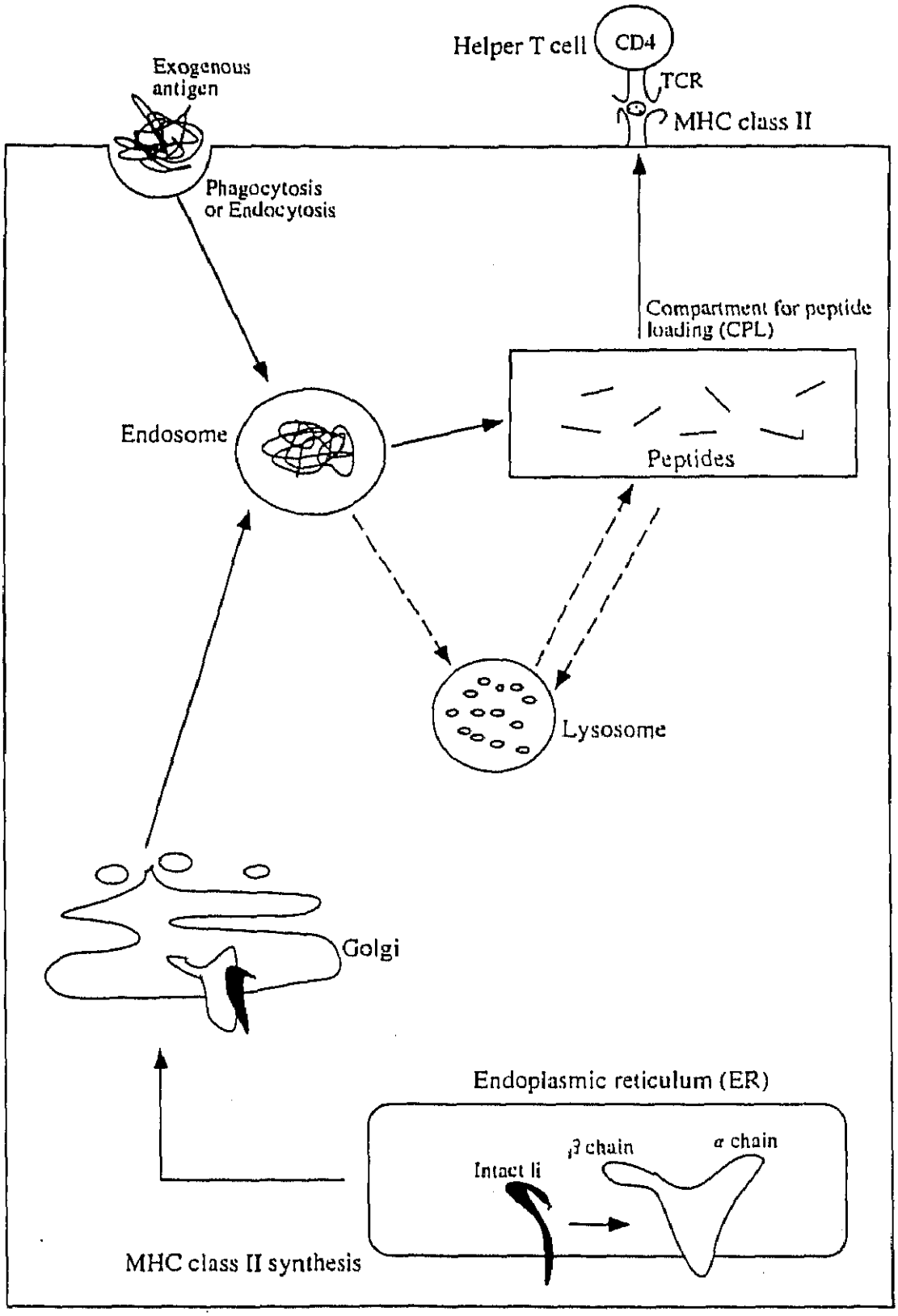


Fig.2a. Pathway of class II MHC-resticted presentatin of an exogenous antigen.

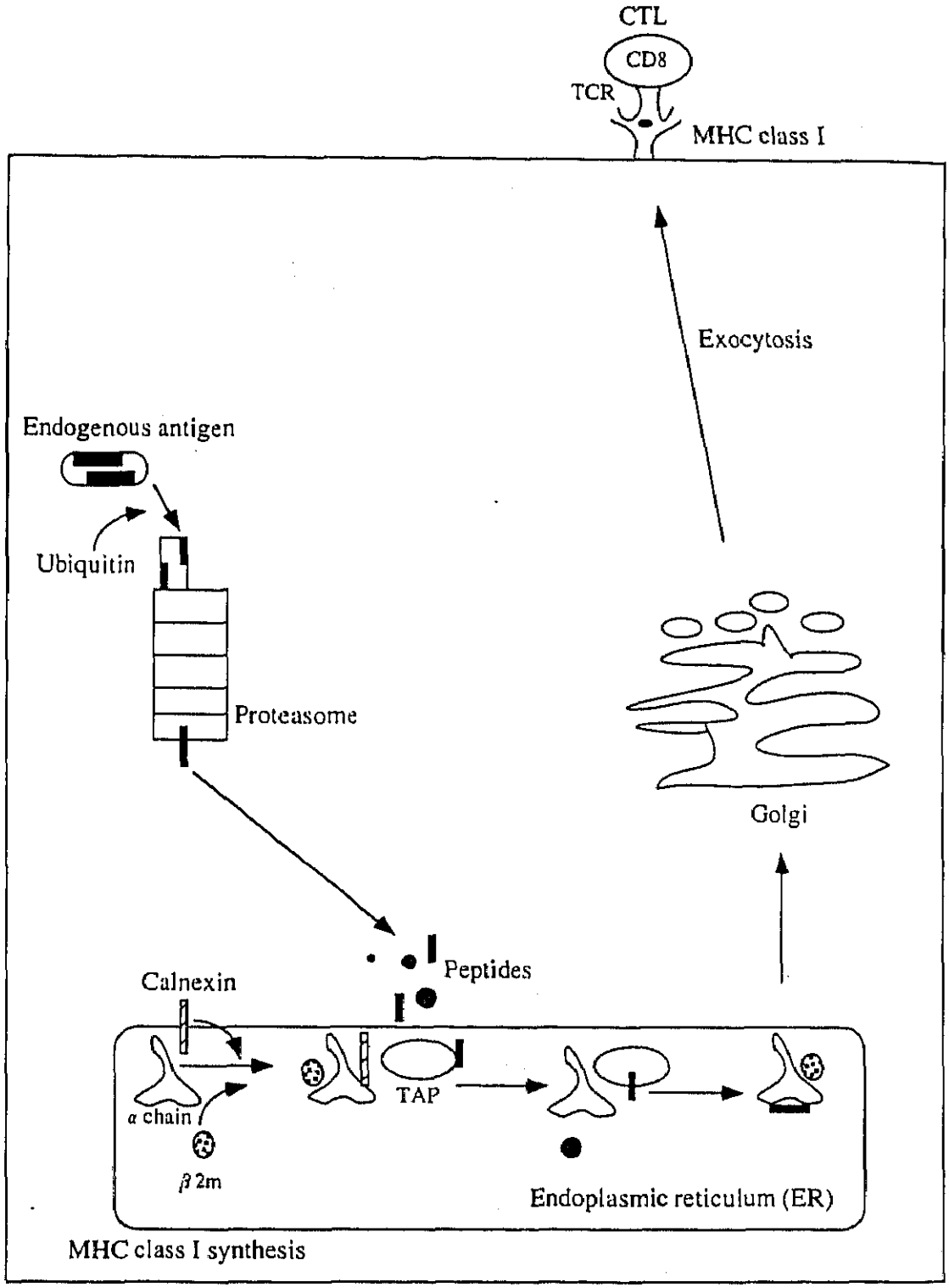


Fig.2b. Pathway of class I MHC-resticted presentatin of an endogenous antigen.

requirement for CTL induction, exogenous soluble antigens do not, in general, induce specific CTL responses. Furthermore, exogenous antigens introduced into the extracellular fluids (as by injection) cannot gain access to the cytosol in most cells and therefore are excluded from the MHC class I processing pathway.

However, recent data show that the stimulation of various types of exogenous antigens efficiently induces MHC class I restricted CTL responses [96-101]. These reports suggested that exogenous particulate antigens are processed and presented by antigen presenting cells (APC) to CTL.

To initate a CTL response, MHC-bound peptides have to be presented to naive T cells by professinoal APC that express high levels of MHC-class I molecules, adhesion receptor, and potent costimulator activity. APC internalize antigen locally and move to lymphoid tissues in two functionally distinct phases: uptake/transportation and T cell activation regulated by inflammatory stimuli TNF- α , IL-1 and LPS [102,103]. The major APC for the generation of CTL responses are macrophages (MΦ) and dendritic cells (DC) [104-108].

Macrophages ingest large particles by phagocytosis, involving cytoskeltal elemenys such as tubulin and actin for the formation ofhighly proteolytic phagosomes [109]. Macrophages can also collborate with DC by degradation and transfer of smaller antigenic

particles to these [110,111]. Recent studies demonstrate that some macrophages have the capacity to process and present exogenous particulate antigens with MHC class I molecules [112-114]. In these antigen-presenting cells (APC), antigens in phagolysosome gain acess to the cytosol and subsequently proteins for MHC class I presentation or in some cases antigenic peptides are regurgitated and bind to surface MHC class I molecules.

For example, OVA (ovalbumin) conjugated to latex-beads can be processed for presentation by MHC class I molecules and can elicit MHC class I restricted T cell responses both in vivo and in vitro [108]. These are all particulate antigen preparations that are efficiently internalized by macrophages, and in vitro studies have demonstrated that phagocytic processing by macrophages is essential in the case of large synthetic particles. Macrophages seem to be the primary cell type that express the aiternate MHC class I processing pathway for presentation of exogenous particulate antigens by MHC class I molecules [113,114]. It is conceivable that these APC may be important participants in the regulation of host immune responses.

The presentation of soluble antigens by the exogenous pathway of phgocytes requires high concentrations of protein [97,115]. However, when the antigen is bound to micro-sized particles, its presentation is enhanced 1000-10000 fold [114]. This effect is due to internalization of

the particles by phagocytosis, as shown by the ability to block presentation of particulate antigens by cytochalasins, which are inhibitors of phagocytosis [108,114], and the failure of nonphagocytic cells to present particulate antigens [114].

Many different type of particles (e.g. liposome-associated, or beads made of latex, ISCOM-associated) can target antigen to the MHC class I pathway of phagocytes [108,116,117]. Recently, soluble proteins incorporated into certain liposome formulationa may permit CTL induction. For example, soluble OVA encapsulated in pH-sensitive liposomes were shown to induce MHC class I-restricted CTL responses in mice [118]. In a subsequent study, it was shown that soluble OVA encapsulated in pH-sensitive liposomes could deliver the antigen to dendritic cells. Dendritic cells cultured in the presence of OVA encapsulated in pH-sensitive liposomes could induce primay CTL in vitro [100]. Furthermore, studies have also reported that macrophages can induce a CTL respose efficently in vitro when particulate carriers are used to deliver antigenic short peptide and whole protein antigen [119]. This report showed that the improvement in presentation of peptide to CTL using latex beads as delivery agent is mediated by macrophages in a process that required phagocytosis of the beads and presumably loading of MHC class I in the phagosomal pathway. These studies results suggested that a useful tool for CTL response induction

in vitro, an important step towards CTL immunothreapy. In addition, it is conceirable that a key event in an immune response is how efficently antigen-presentation cells (APC) pick up and process antigens and present processed antigens to T lymphocytes.

If autologous CTL specific to tumor cells that produce soluble and/or membrane-bound antigen could be induced, this would have strong implication for the development of adoptive immunotherapy for the tumor patient. Carcinoembryonic antigen (CEA) is a well-known soluble tumor marker frequently detectable in peripheral blood of carcinoma patients. Elevated level of CEA has been detected in the blood circulation of patients with colon cancer, some types of lung cancer, pancreatic cancer, and breast and stomach cancer [120,121]. 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 [122]. Human CTLs response against CEA-producing cells was observed in the patients injected with the recombinant vaccinia-CEA vaccine [123]. We have investigated whether it is possible to induce in vitro human CTLs responding to the CEA-producing cancer cells by utilizing fixed macrophage-rich peripheral blood cells pre-loaded with particulated form of CEA whole protein. Results suggest that the CTLs recognized HLA-A2402 restricted peptides derived from CEA.

Material and Methods

Target cells and culture condition

We selected three cell lines with the same MHC-class I subtype HLA-A2402, namely, a gastric adenocarcinoma cell line MKN45 that is poorly differentiating and well known to produce a high level of CEA [124] and, as controls, a stomach adenocarcinoma cell line GT3TKB and kidney adenocarcinoma cell line Hpt.10. The HLA subtype was determined by the method of Blasczyk et al. [125]. Together with these cell lines, Daudi cells and K562 cells were provided from RIKEN Cell Bank. All the cell lines were maintained in RPMI-1640 medium (see Table. 1) supplemented with 10% fetal bovine serum (FBS) in an atmosphere of humidified 5% CO_2 in air.

Reagents

CEA prepared from a metastasis of human colon cancer was purchased from Chemicon International Inc. (Catalog number AG11P); anti-human CEA monoclonal antibody (180 kD, clone PARLAM4, Catalog number MON 9008), Sanbio BV; polybeads carboxylate dyed microparticles (2.5% solids-latex, Catalog number 19816), Polyscience Inc.; 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 (w6/32 clone), Dako Japan Inc.; anti-human MHC-class II monoclonal antibody (CR3/43 clone), Dako Japan Inc. The set of 10 HLA-A24 epitope peptides of CEA and a control irrelevant peptide (Code No. SP102, Lot No. 0011, see Fig. 9 for the sequences) was purchased from Biotech Research Lab., Takara Shuzo Co., Ltd, Ohtsu, Japan. Sequences of the CEA peptides were derived from carcinoembryonic antigen precursor (Swiss Prot: P06731, see Table 2). The amino acid sequence of CEA (GeneBank Accession M17303) was scanned for peptides having a length of 9 or 10 amino acids, containing the HLA-A24 binding motif (Y, F or W at position 2 and F, L, I or W at the C-terminus). The control irrelevant peptide was derived from a nucleoprotein of influenza virus (Swiss Prot: P18277). The CEA peptide (TLSGANLNL) was purchased from Biologica Co., Japan. This peptide was designated as CAP-1 and is the HLA-A2 restricted CTL epitope [123]. Genomic typing of tumor cells and lymphocytes has been carried out commercially by Tokyo Laboratory, Shionogi & Co., Ltd. (Tokyo, see Table 3).

Table 1. Composition of RPMI-1640 medium (in 10.2g for 1 liter)

L-Arginine Hydrochloide	240mg	Riboflavine	0.2mg
L-Asparagine, H_2O	56.8mg	Thiamine Hydrochloride	1mg
L-Aspartic Acid	20mg	Cyanocobalamine	0.005mg
L-Cysteine Hydrochloride, H_2O	72.9mg	Succinic Acid	46mg
L-Glutamic Acid	20mg	Sodium Succinate, $6H_2O$	0.612mg
Glutathione	1mg	Sodium Chloride	6000mg
Glycine	10mg	Potassium Chloride	400mg
L-Histidine Hydrochloride, H_2O	20.3mg	Calcium Nitate	69.5mg
L-Hydroxyproline	20mg	Monosodium Phosphate	677mg
L-Isoleucine	50mg	Magnesium Sulfate	48.8mg
L-Leucine	50mg	Glucose	2000mg
L-Lysine Hydrochloride	40mg		
L-Methionine	15mg		
L-Phenylalanine	15mg		
L-Proline	20mg		
L-Serine	30mg		
L-Threonine	20mg		
L-Tryptophan	5mg		
L-Tyrosine	20mg		
L-Valine	20mg		
Phenol Red	5mg		
Biotin	0.2mg		
Calcium Pantothenate	0.25mg		
Choline Chloride	3mg		
Folic Acid	1mg		
i-Inositol	35mg		
Nicotinamide	1mg		
p-Aminobenoic Hydrochloride	1mg		

Table 2. List of HLA-A24(A*2402)binding peptides from CEA

Peptide	aminoacid	sequence	A*2402binding (IC50,nM)
CEA10	10	RWCIPWQRLL	151.0
CEA101	9	IYPNASLLI	0.84
CEA234	9	LYGPDAPTI	28.0
CEA268	10	QYSWFVNGTF	1.73
CEA318	9	VYAEPPKPF	20.0
CEA425	9	TYYRPGVNL	89.0
CEA426	10	TYYRPGVNLSL	42.0
CEA590	9	LYGPDTPII	45.0
CEA604	10	SYLSGANLNL	101.0
CEA652	9	TYACFVSNL	4.96

Table 3. Genomic typing of tumor cells

Target cell	Tissue	CEA	HLA-typing*
MKN 45	Stomach	Positive	HLA-A2402
			HLA-B5201
			HLA-Cw1202
GT3TKB	Stomach	Negative	HLA-A2402
			HLA-B5201
			HLA-Cw1202
Hpt. 10	Kidney	Negative	HLA-A2402/0206
			HLA-B5401/3901
			HLA-Cw0102/0702

* Genomic typing of tumor cells has been carried out commercially by Tokyo Laboratory, Shionogi & Co., Ltd. (Tokyo).

Preparation of human monocytes-derived macrophages (M Φ) and phagocytosis

Human monocytes were prepared by the conventional Ficoll-Paque centrifugation method from heparinzed peripheral blood of a healthy volunteer. The cells were suspended in RPMI 1640 medium containing 10% FBS at a cell density of $5x10^5$ cells/ml, and added to tissue culture dishes was formed by attachment of a coverglass, which was then incubated at 37 °C for 1hr.

Nonadherent cells, including containing lymphocytes, were then washed off, and adherent monocytes were further incubated for 2hr at 37 °C in the tissue culture dishes with 3 ml medium containing fluorescent latex beads. As control, no addition was used. After 2hr incubation, the adherent cells were fixed with methanol for 20 mim at room temperature. The phagocytosis of macrophages was confirmed by fluorescent microscopy.

CEA binding to latex beads

We used the carbodiimide kit (Polyscience, Inc.) for covalent coupling of CEA to carboxylated microparticles. Briefly, after placing 0.5 ml of the latex bead suspension in a 1.5-ml Eppendorf centrifuge tube, the tube was filled with the carbonate buffer provided in the kit, then centrifuged for 6 min at 10,000 rpm in a microcentrifuge. The pellet

was washed once with fresh carbonate buffer and once with the phosphate buffer and then resuspended in the phosphate buffer. To the suspension, 0.6 ml of phosphate buffer and 0.6 ml of 2% carbodiimide solution were added and incubated for 4 hr at room temperature with gentle end-to-end mixing and then pelletted by centrifugation. To activate the beads, this suspension was placed in a well of a 24-well culture plate, irradiated with short wavelength UV light overnight, and pelletted by centrifugation.

A hundred μg of CEA was mixed with one ml of borate buffer, then added to the activated bead suspension and left overnight with gentle mixing at room temperature. The beads coated with CEA were once pelletted, resuspended in 1ml of 0.1M ethanolamine, mixed gently for 30 minutes at room temperature, pelletted again, and then resuspended in 1 ml of PBS. This CEA-bead suspension was used as the CEA-beads for incubation with the adherent cells obtained from human PBMC.

Preparation of antigen presenting cells and pulsing CEA-beads

Human PBMC were prepared by the conventional Ficoll-Paque centrifugation method from heparinized peripheral blood of a healthy volunteer who has been identified as carrying HLA-A-2402 subtype. The cells were washed once with PBS, then once with RHAMa medium (see Table 4) supplemented with 5% autologous plasma and

centrifuged by centrifugation at 1400 rpm for 10 min at room temperature as previously described [126].

The separated PBMC (2x106 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.

Table 4. Compornent of RHAM- α (mg/L)

L-Alanine	6.78mg	Folic acid	1.064mg
L-Arginine	187.42mg	Inositol	23.004mg
L-Asparagine	47.08mg	Nicotinamide	0.808mg
L-Aspartate	20.66mg	Pyridoxal	0.2mg
L-Cysteine	27.02mg	Pyridoxine	0.612mg
L-Cystine	34.8mg	Riboflavin (B2)	0.148mg
L-Glutamine	267.64mg	Thiamin (B1)	0.868mg
L-Glutamate	29.94mg	NaCl	6480mg
Glycine	17.5mg	KCl	367.74mg
L-Histidine	21.58mg	$CaCl_2$	106.66mg
L-Isoleucine	41.28mg	$CuSO_4 \cdot 7H_2O$	114.44mg
L-Leucine	43.12mg	NaH_2PO_4	28mg
L-Lysine	45.9mg	Na_2HPO_4	508.82mg
L-Metionine	12.88mg	$NaHCO_2$	1835.2mg
L-Phenylalanine	16.6mg	$CuSO_4 \cdot 5H_2O$	0.0004mg
L-Proline	26.9mg	$ZnSO_4 \cdot 7H_2O$	0.172mg
L-Hydroxyproline	12mg	$FeSO_4$	0.092mg
L-Serine	25.1mg	Glucose	1760mg
L-Threonine	23.9mg	Glutathione	0.6mg
L-Tryptophan	5.44mg	Pyruvate	44mg
L-Tyrosine	20.32mg	Lipoic acid	0.082mg
L-Valine	23.72mg	Putrescinedichloride	0.032mg
Para-aminobenzoic acid	0.6mg	Hypoxanthine	0.816mg
Ascorbate (C)	10mg	Thymidine	0.186mg
Biotin (H)	0.154mg	Linoleate methyl	0.018mg
Pantothenate	0.446mg		
Choline	4.8mg		
Cyanocobalamin (B12)	0.555mg		

The adherent cells with monocytes/macrophages-like morphology were further cultured overnight in the 24-well plate with 2 ml of the medium containing CEA-beads (50 μg). As controls, CEA protein alone (50μg/ml), none, or 50 μg of naked latex beads was used. After 24 hr incubation, the adherent cells were fixed with 10% (v/v) formalin in PBS for 1 hr at room temperature and washed thoroughly with the culture medium before the induction of CTLs.

Induction of CTLs from PBMC

On the fixed adherent cells, PBMC ($1x10^6$ cells) freshly prepared from the same volunteer were added with 2 ml of RHAMa medium supplemented with human interleukin(IL)-1β (Otsuka Pharmaceutical Co., Ltd., 167 U/ml), IL-2 (Shionogi & Co., Ltd., 67 U/ml), IL-4 (Ono Pharmaceutical 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 volume of medium change every other day. The effector cells were re-stimulated weekly to a total of 3 re-stimulation at an effector: APC ratio of 2. The fixed adherent cells were renewed by transferring the cultured PBMC once a week (see Fig. 3). In addition, CEA-peptide specific CTLs were generated by re-stimulating the PBMC 3 times with the fixed adherent cells previously pulsed with CEA652(9) peptide (TYALFVSNL, 50 μg/ml) for 1 hr at 37 °C.

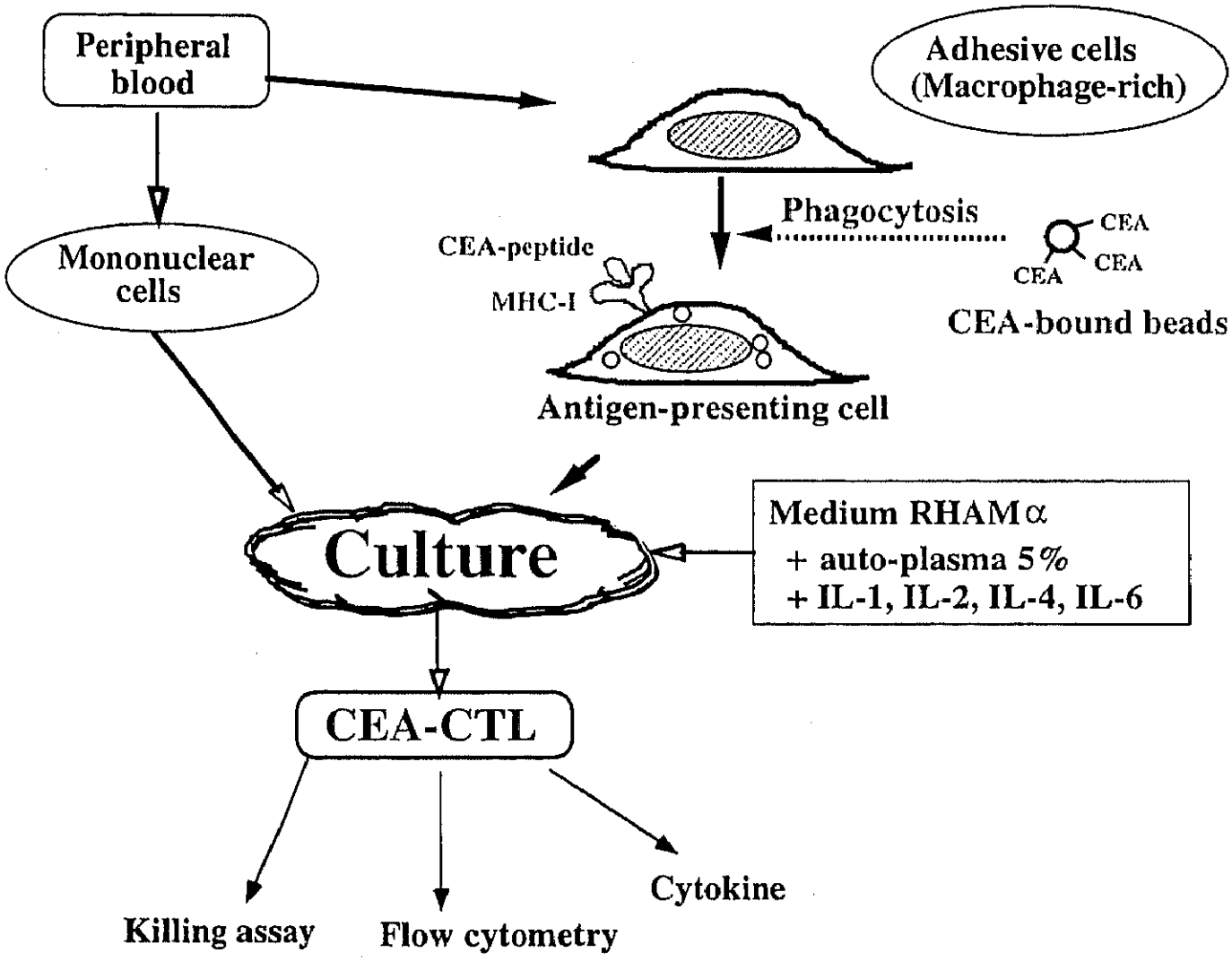


Fig.3. Induction of Autologous CEA-CTL

Staining of cell surface CEA and flow cytometry

The target cancer cells ($1x10^6$) were washed three times with calcium-, magnesium-free PBS (PBS(-)), stained for 30min with the monoclonal antibody against CEA, and then stained for 30min with FITC-labeled goat anti-mouse IgG polyclonal antibody. The cells were again washed three times with PBS(-) containing 4% FBS. They were resuspended in the same buffer at a concentration of 1x106 cells/ml and were immediately analyzed using FACScan (Becton-Dickinson, Co.) as has been previously described [65]. Proportion of $CD3^+$, $CD4^+$, $CD8^+$ cells were determined in the same manner using corresponding monoclonal antibodies.

Cytotoxicity assay

Assay for cell-mediated killing of target cancer cells was performed in vitro using the crystal violet (CV) staining method as described previously [65, see Fig. 4]. This CV assay used in the present study is as sensitive for assessment of the killing activity of $CD8^+$ T lymphocytes as the standard ^{51}Cr-release cytotoxicity assay [65]. Briefly, the target cells, $1x10^4$ cells/well in 200μl RPMI 1640 medium containing 5% FBS, were seeded in each well of 96-well plates and were precultured for 12 hrs. The cultured target cells were washed once with PBS(-), then, the cultured PBMC suspended in 200μl of RHAMa containing 5%

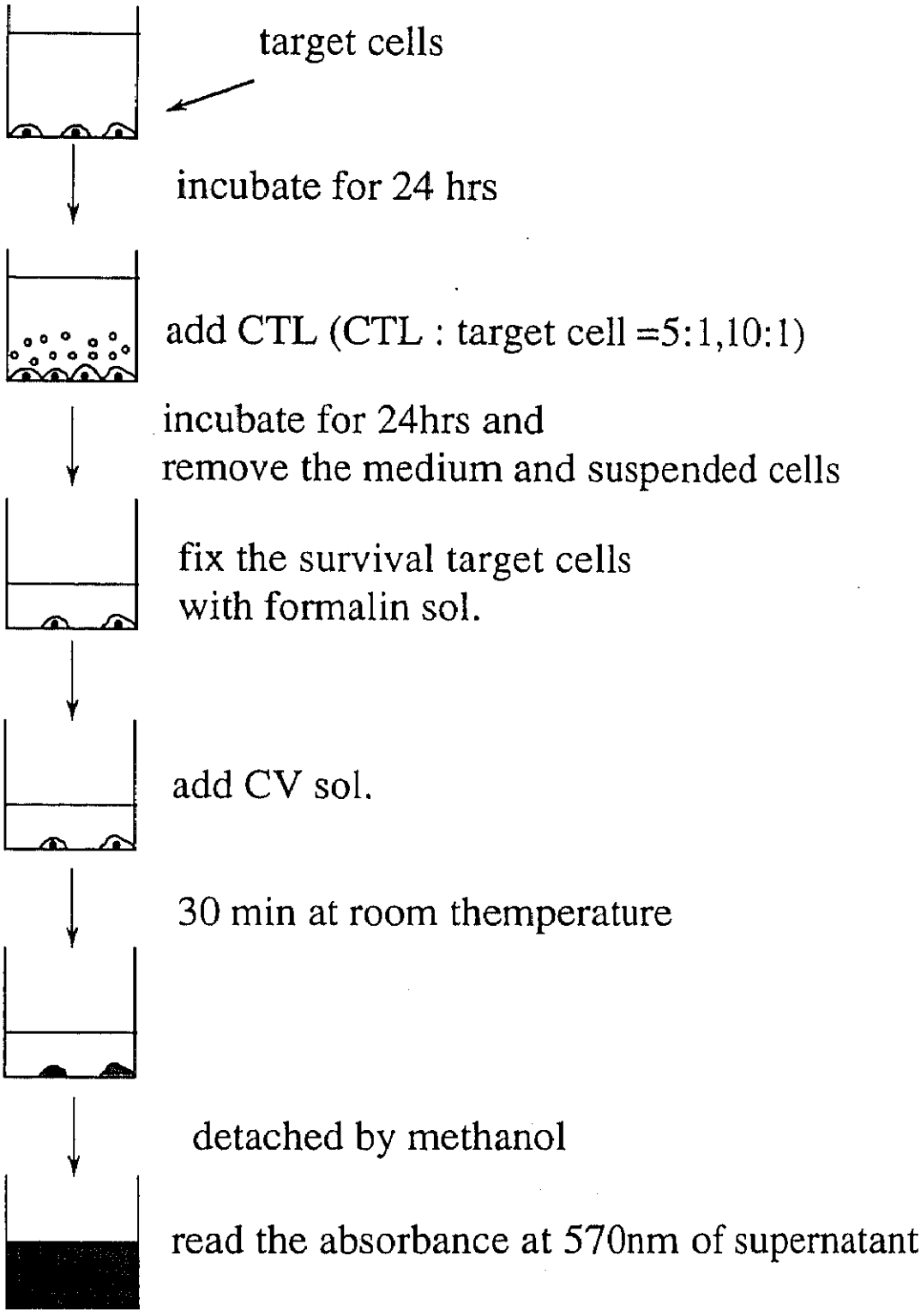


Fig. 4. CV staining method

autologous plasma were added as effector cells to each well at the indicated effector to target ratio (E/T ratio). The cells were cocultured for 24 hrs.

The wells were washed once gently with appropriate amount of PBS. Adherent target cells were fixed for 1 hr with 10% (v/v) formalin (200 μl/well), then stained with crystal violet solution (0.4% in water, 100 μl/well) for 30 min at room temperature. The plate was washed with tap water and dried at room temperature. To each well, 200 μl of 80% methanol was added and the OD570 of each well was determined. As 100% control, we determined the OD570 of the target cells precultured in a separate plate just before the addition of the effector cells.

Percentage of surviving target cells was defined as follows:

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

where A is the OD570 of the well containing the target cells and the CTLs, B is the OD570 of the 100% control well of the target cells, and C is OD570 of the well containing medium alone.

In the present experiments, the wells containing the CTLs alone showed almost the same OD570 as C, or if any slightly high OD570, even in the wells corresponding to E/T of 10. Therefore, we thought that the remaining lymphocytes in the wells after the 24 hr coculture were sufficiently washed out. Marginal amount of the lymphocytes may have remained attached on the target cell layer, but they were included as the

survived target cells. Therefore, underestimation, though very slight, of the killing activity of the CTLs should be considered in this assay. Each value in the figures represents the mean of four replicates accompanied with an error bar of SD. Note that the carcinoma cells cultured at E/T = 0 grew vividly for the 24 hr incubation and, therefore, showed more than 200% of surviving.

For testing CTL recognition of the HLA-A2402-binding peptide derived from CEA, GT3TKB target cells were incubated with each peptide at a final concentration of 50 μg/ml for 1 hr at 37 °C, washed once with the culture medium, and then the effector cells were added and cocultured for 24 hr. To detect activities of natural killer cells or lymphokine-activated killer cells, WST-1 assay was performed in the 24 hr coculture of the effector lymphocytes and the target K562 cells and Daudi cells as described [65]. In brief, four hours before the end of incubation, 20 μl WST-1 dye solution (65 mg WST-1, 7 mg 1-methoxy WST-1 in 100 ml of PBS, Wako Chemicals, Co., Tokyo) was added directly to the the 96-well plates. The absorbance at 450nm was measured. Percentage of surviving target cells was defined as follows:

$$\text{Surviving target cell}(\%)=(B-C)/Ax100$$

Where A is the absorbance of control target cells without CTL at the end of incubation, B is that of remaining target cells to which CTL were added, and C is that of CTL only.

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 [65]. These antibodies were used at a final concentration of 5 μg/ml. Target MKN45 cells were precultured overnight in 96-well plates at 1x104 cells/well. These target cells were pretreated also as described [65] with antibodies against human MHC-class I or MHC-class II at a final concentration of 10 μg/ml and then incubated with the effector cells at 37 °C for 24 hr. The adherent target cells were quantified by the CV-staining method [65]. As 100% control, we determined the OD570 of the target cells precultured in a separate plate just before the addition of the effector cells. The inhibition of cytotoxic activity was calculated according to following formula:

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

where A is OD570 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 OD570 value of the target cells to which the effector cells were added (both of the cells were not pretreated with any monoclonal antibodies; and T is OD570 value of the target cells to which neither CTL nor antibodies were added (OD570 of 100% control).

Enzyme-linked immunosorbent assay (ELISA)

For detremination of cytokine production, the induced effector cells ($1x10^6$/well) were plated into 24-well plates with CEA beads-pulsed APC ($2x10^6$/well). The culture supernatants were collected 24 hr later. The levels of IFN–γ and TNF–α in the culture supernatants were assessed by two-site ELISA according to the manufacturer's protocol (phaMinger, San Diego. CA). In brief, to IFN–γ , plates (MaxiSorp; NUNC, Roskilde, Denmark) were coated with anti-IFN–γ mAb NIB42 and IFN–γ bound was detected by means of biotinylated anti-IFN–γ mAb 4S.B3. To detect TNF–α, anti-TNF–α mAb MAb1 and biotinylated anti-TNF–α mAb MAb11. were used as the coating antibody and detecting antibody, repectively. The results were expressed in pg/ml.

Results

Phagocytosis of macrophages

We investigated whether human monocytes-derived macrophages (MΦ) could phagocytose latex beads. As shown in Fig. 5, we confirmed that latex beads were phagocytosed by macrophages.

CEA expression on the target tumor cells

We have confirmed expression of CEA on the surface of the cell lines MKN45, GT3TKB, and Hpt.10. These cell lines were selected because they belong to the same MHC-class I subtype, HLA-A-2402. As shown in Fig. 6, the expression of CEA was apparent on the cell surgace of MKN45 (Fig. 6a), but not on GT3TKB (Fig. 6b) and Hpt.10 (Fig. 6c) cells.

Growth characteristics of the lymphocytes cultured on the fixed adherent cells

Fig. 7 shows growth curves of the lymphocytes derived from a healthy volunteer whose MHC-class I subtype has been identified as HLA-A2402/3303, B3501/44031, Cw0303/1403. His peripheral blood adherent cells containing macrophages have been previously loaded

with CEA-beads and fixed, then incubated with live autologous PBMC as described in Materials and Methods. Although the adherent cells were fixed with formalin, the PBMC gradually degraded the adherent cell layer in the induction culture of CTLs. Therefore, the lymphocytes were restimulated weekly on the refreshed fixed adherent cell layer. After 14 days, the number of lymphocytes increased on the fixed cell layer previously loaded with the CEA-beads, but only slightly on the fixed cells previously loaded with control CEA protein and control naked beads as well as medium alone.

Cytotoxic activity of the cultured lymphocytes

We have confirmed expression of CEA and MHC-class I molecules on the surface of the cell lines MKN45 (expressing HLA-A2402, B5201, Cw1202), GT3TKB (HLA-A2402, B5201, Cw1202), and Hpt.10 (HLA-A0206/2402, B3901/5401, Cw0102/0702). MKN45 and GT3TKB cells exhibited the same HLA-pattern observed in more than 30% of Japanese (Tokyo Laboratory, Shionogi & Co., Ltd., personal communication). Each HLA-allele of these two cell lines may be the same or half-defective, although these were apparently different origin confirmed by RIKEN Cell Bank. More than 99% of the target MKN45 cells expressed high levels of CEA as well as MHC-class I molecules, but the expression of CEA was not detectable on GT3TKB and Hpt.10

cell (Fig. 8a, 8b and 8c).

We used CV staining assay [65] for the observation of target cell killing by the lymphocytes in the 24-hr coculture where OD570 of the target cells at the start of the coculture were taken as 100%. Since the target cells grew during the 24-hr incubation, the percentage of the surviving target cells exceeded 100% as shown in Fig. 5a-c. We assumed that, if the surviving target cells did not decrease to less than 100%, the lymphocytes may have simply inhibited the growth of the target cells [126].

When the lymphocytes were tested for their cytotoxic activity on live target carcinoma cells 7 days after the last re-stimulation, apparent killing was observed only in the combination that contained the lymphocytes stimulated with the fixed adherent cells previously loaded with the CEA-beads and the CEA-producing MKN45 cells (Fig. 8a).

In contrast, the fixed adherent cells previously loaded with or without control beads as well as control CEA protein alone did not generate the killing response of the lymphocytes against CEA-producing MKN45 cells. To the other two carcinoma cell lines, GT3TKB and Hpt.10, the lymphocytes did not show any apparent cell killing activity (Fig. 8b and 8c), although the target cells expressed the common HLA-A2402 on their surface. The lymphocytes showed substantially no killing activity examined by WST-1 assay [65] against natural killer cell-sesitive K562

cells and lymphokine-activated killer cell-sensitive Daudi cells tested at E/T ratios of 5 and 10 (data not shown).

Recognition of CEA epitope peptides by the CTLs

We examined whether the CTLs recognize HLA-A2402-binding peptide derived from CEA. Ten peptides derived from CEA have recently become commercially available (Fig. 9) which were selected on the basis of high binding affinity to purified HLA-A2402 protein [K. Takesako and I. Nukaya, Takara Shuzo Co., personal communication]. Killing activity of the CTLs was examined against target GT3TKB cells pulsed with one of the CEA peptides. As shown in Fig. 9, pulse of the 9-mer peptide CEA652(9) (TYACFVSNL) resulted in the same killing as that observed in the CEA producer MKN45 cells. Unexpectedly, apparent killing was observed in the GT3TKB cells pulsed with a 10-mer peptide CEA10(10) (RWCIPWQRLL) of which sequence locates in the N-terminal leader peptide of the CEA precursor protein. The other 8 peptides revealed lesser antigenic activity than these two peptides in the killing assay, while the control irrelevant peptide, FLU38(10) (RFYIQMCTEL), derived from a nucleoprotein of an influenza virus, showed no antigenic activity to the CTLs ($P<0.01$ or $P<0.05$ for target cells pulsed with each CEA peptide compared with target cells pulsed with the irrelevant control peptides, FLU38(10),

according to the Student's t-test). Furthermore, no killing activity of the CTLs was observed against the target cells that pulsed with CEA-derived HLA-A2(but not HLA-A2402)-restricted CAP-1, the peptide 571(9) (YLSGANLNL).

We also found that blood adherent cells pulsed with the HLA-A-2402-restricted peptide, CEA652(9), and then fixed with formalin, generated the CEA-specific CTLs from PBMC in vitro (Fig. 10). The PBMC and the adherent cells used in this experiment were from the same healthy donor carrying HLA-A-2402. The CTLs could kill CEA652(9)-pulsed GT3TKB cells as well as MKN 45 tumor cells that were expressing the CEA antigen, but not the GT3TKB cells that were not pulsed with the CEA peptide.

Phenotype of the CTLs

We next determined the phenotype of the CTLs generated on the fixed adherent cells loaded with CEA-beads. As shown in Table 5, they consisted of $CD3^+$ cells, 98.3%; $CD4^+$ cells, 2.2%; $CD8^+$ cells, 72.9%. The lymphocytes generated on the fixed cells previously pulsed with the peptide CEA652(9) consisted of $CD3^+$ cells, 97.5%; $CD4^+$ cells, 18.3%; $CD8^+$ cells, 78.6%. Contrary to the low $CD4^+$ and high $CD8^+$ cell proportion, the lymphocytes cultured on the fixed cells loaded with control CEA protein consisted of $CD3^+$ cells,98.4%;

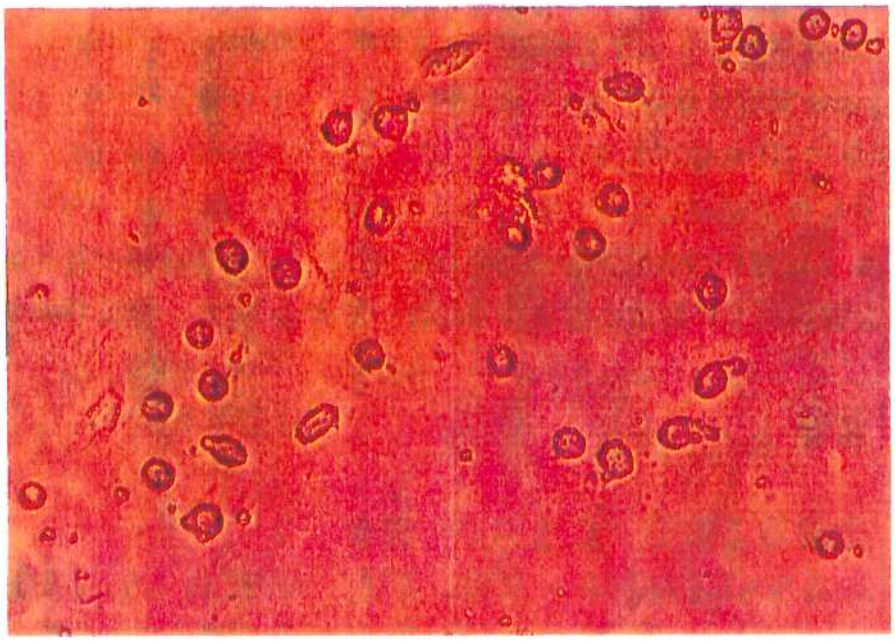
$CD4^+$ cells, 11.0%; $CD8^+$ cells, 29.9%.

Production of cytokines

Since CTL are known to produce cytokines including interferon-γ and tumor necrosis factor-α in an antigen-specific manner, we assayed these cytokines in the culture medium of CTL after stimulation with the autologous fixed adherent PBMC previously loaded with the CEA-containing beads.

As shown in Fig. 11, the effector cells produced these cytokines but showed essentially no cytokine production when stimulated with the fixed adherent cells without CEA-beads pre-loading.

A)



B)

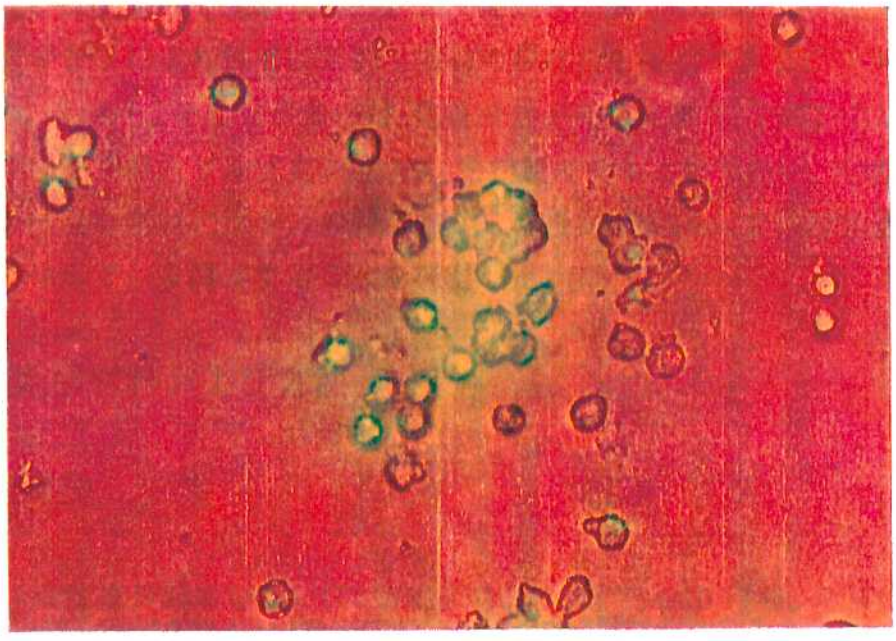


Fig. 5. Phagocytosis of macrophages

(A) Macrophages cultured in standard medium alone.

(B) Macrophages treated with fluorescent latex beads.

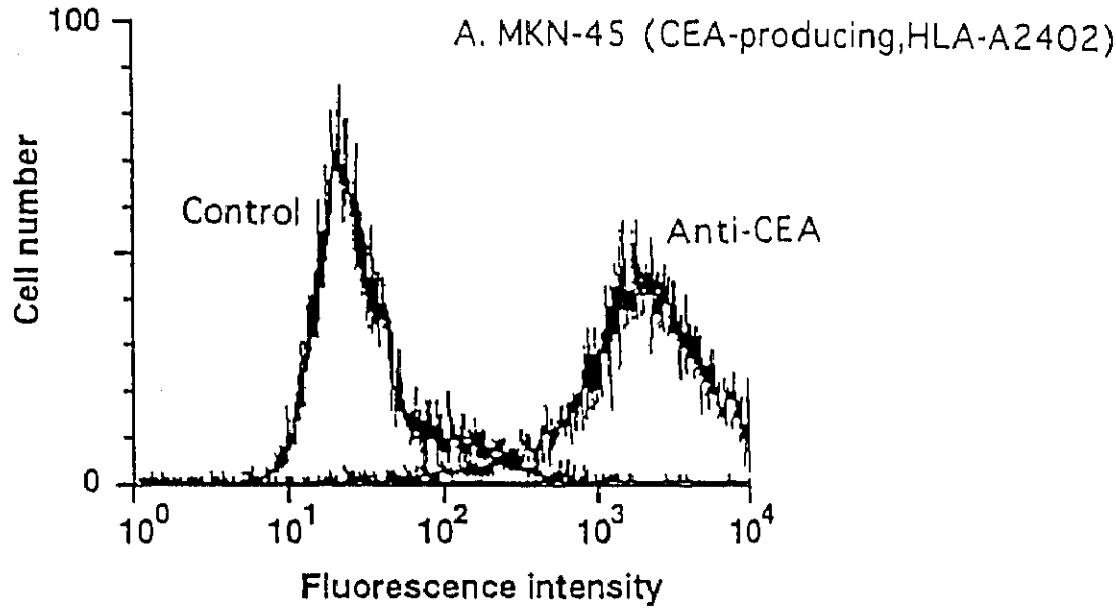


Fig. 6a. CEA expression on MKN-45 target tumor cell

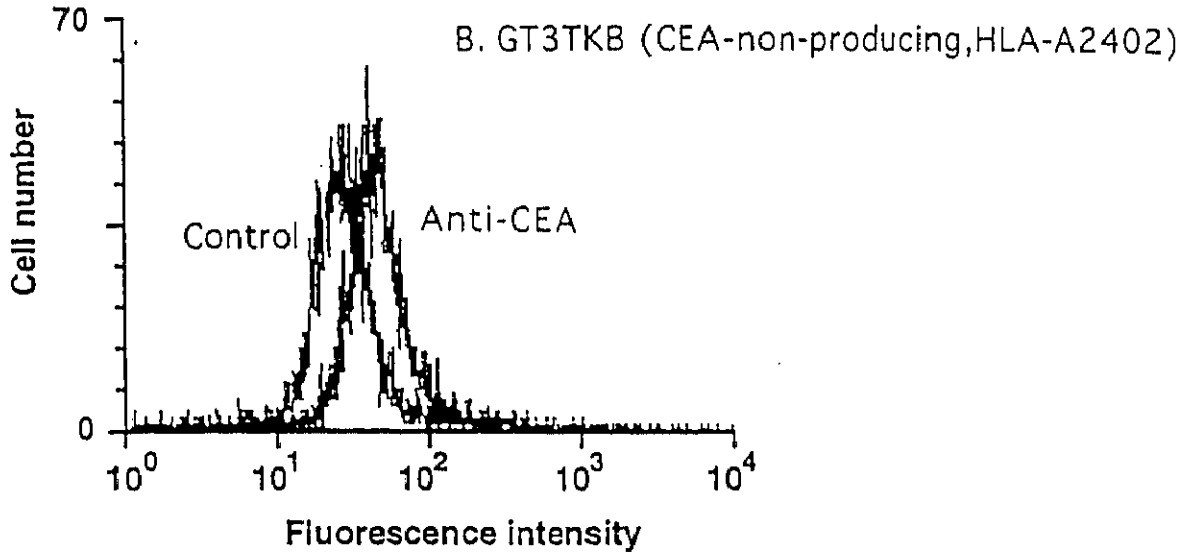


Fig. 6b. CEA expression on GT3TKB target tumor cell

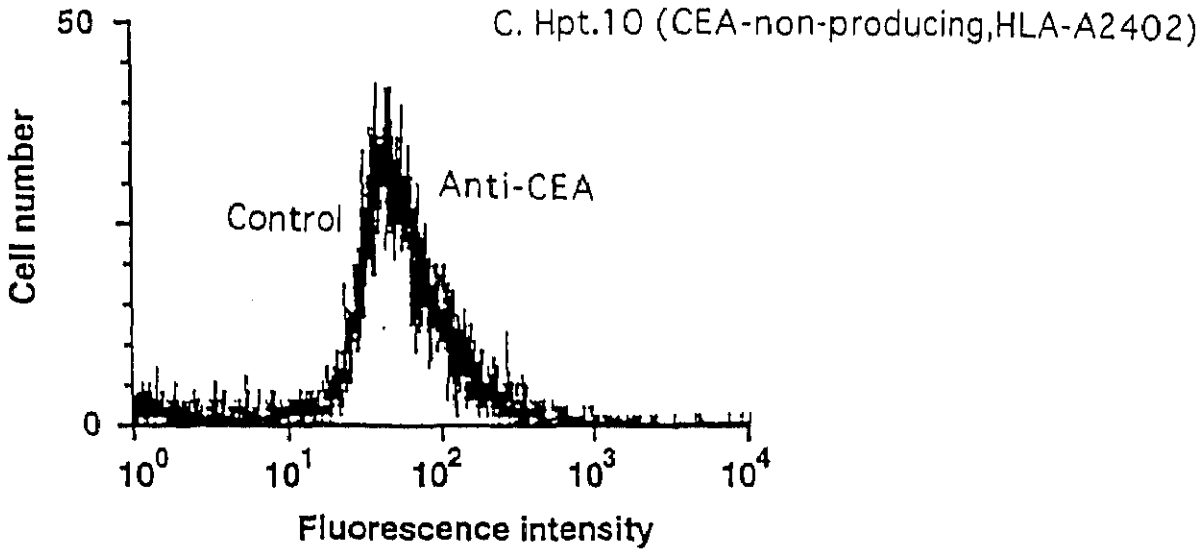


Fig. 6c. CEA expression on Hpt. 10 target tumor cell

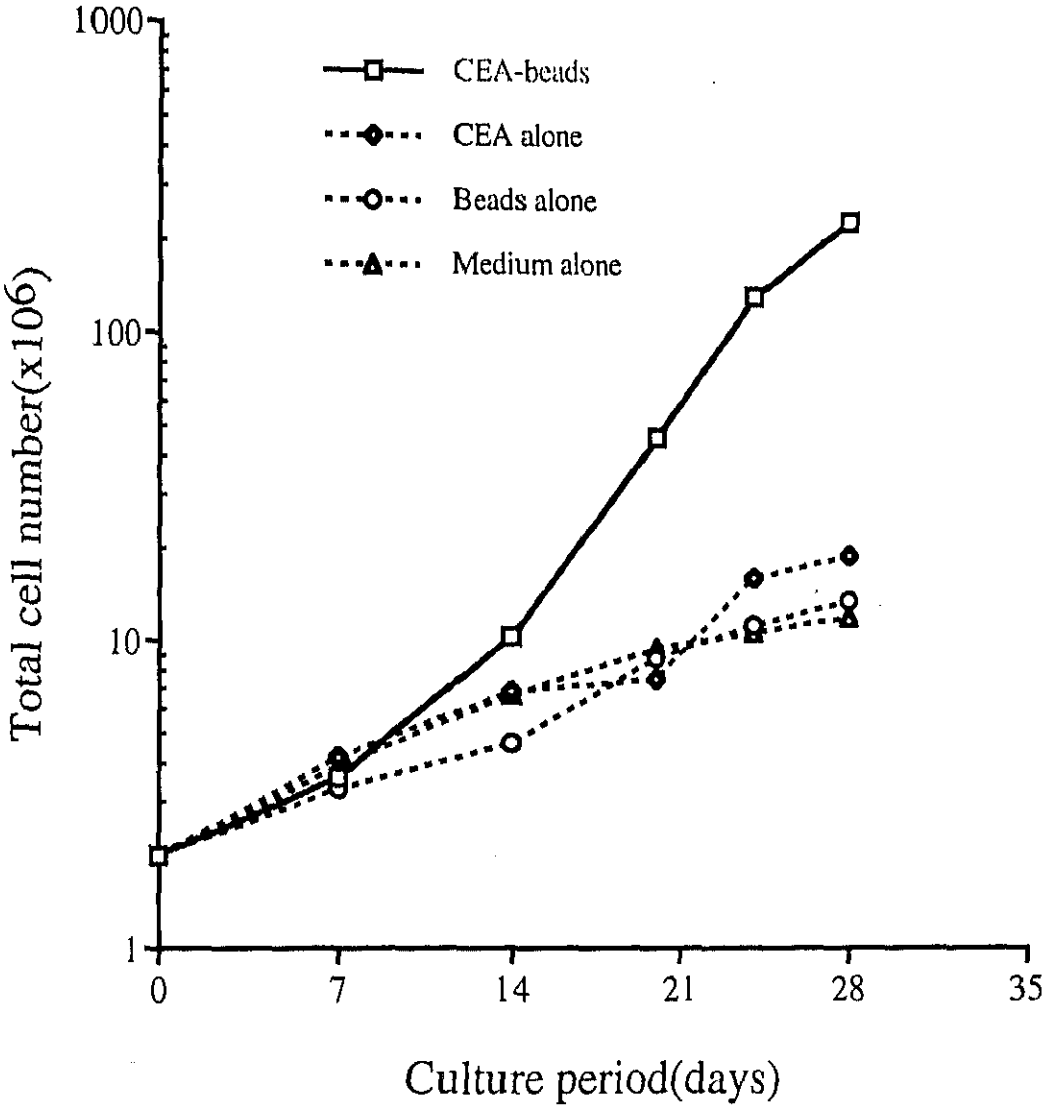


Fig. 7. Cumulative growth curves of the cultured lymphocytes. The cells were cultured with fixed adherent cells pre-loaded with CEA-containing beads, control CEA protein, naked control beads, or no beads (medium alone)

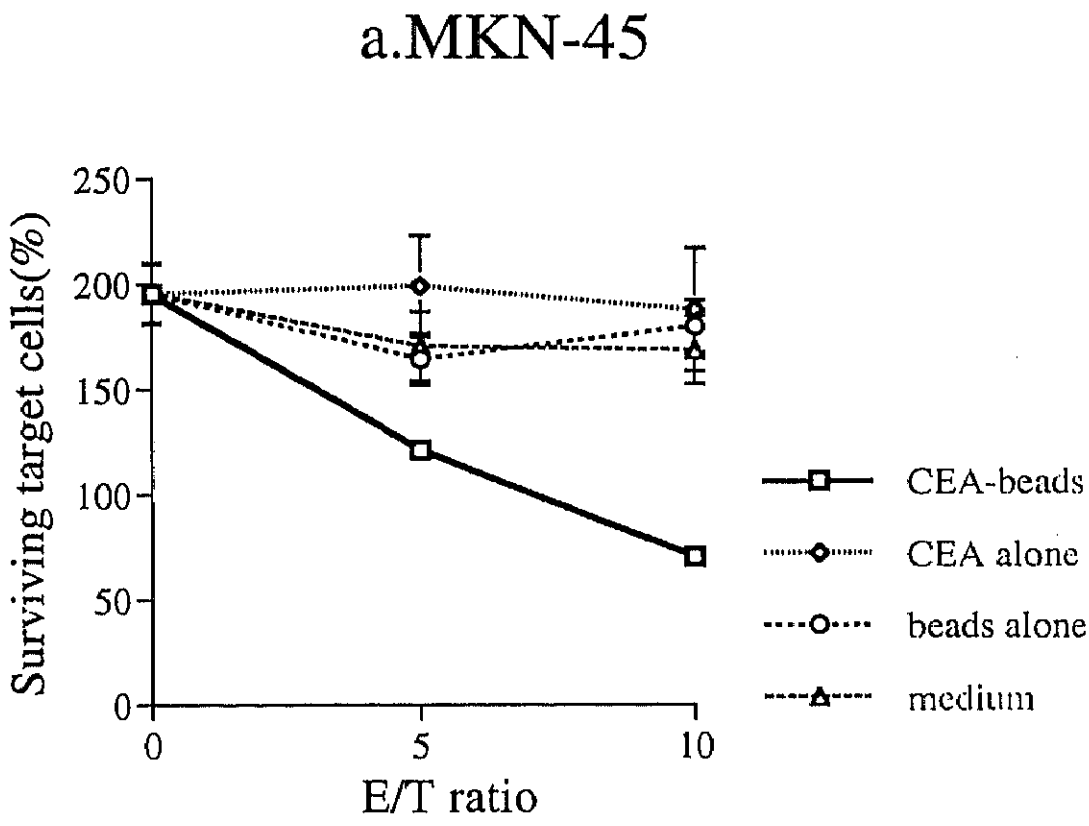


Fig. 8a. Cytotoxicity of the effector cells against MKN45, CEA-positive HLA-A2402 gastric adenocracinoma cells.
The killing assay was performed by CV assay as described in Materials and Methods. Each point represents the mean of four replicates and it accompanied by an error bar indicating SD.

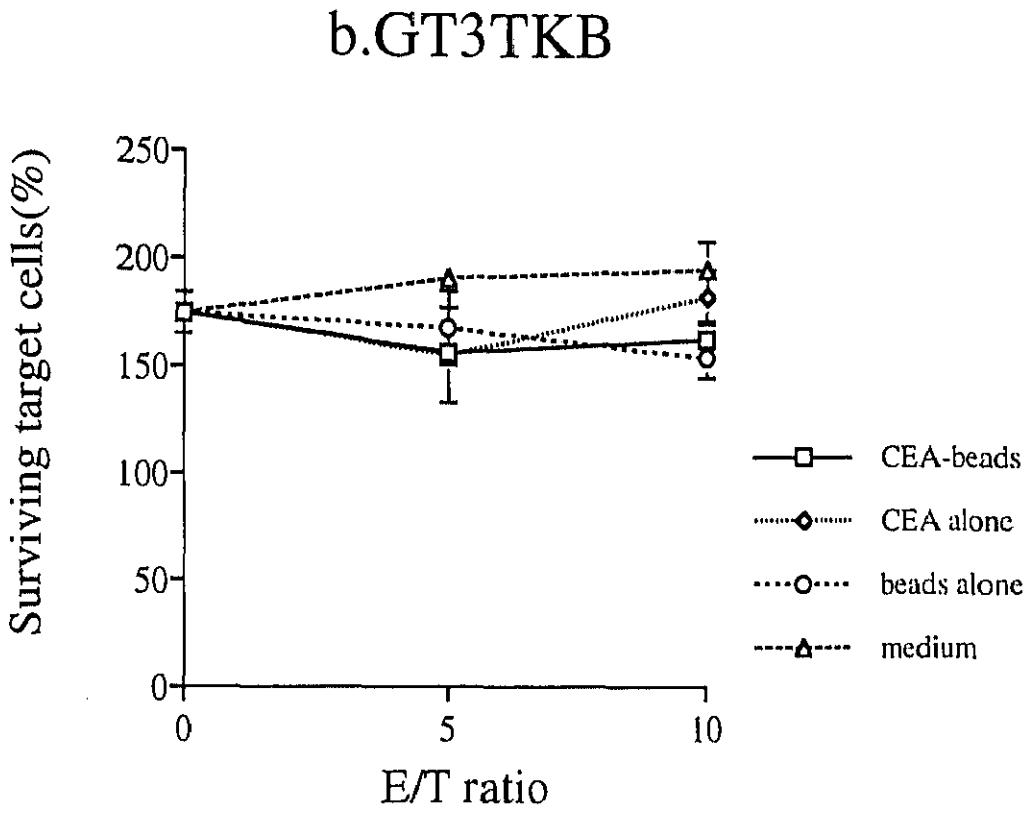


Fig. 8b. Cytotoxicity of the effector cells against GT3TKB, CEA-negative HLA-A2402 gastric carcinoma cells. The killing assay was performed by CV assay as described in Materials and Methods. Each point reoresents the mean of four replicates and it accompanied by an error bar indicating SD.

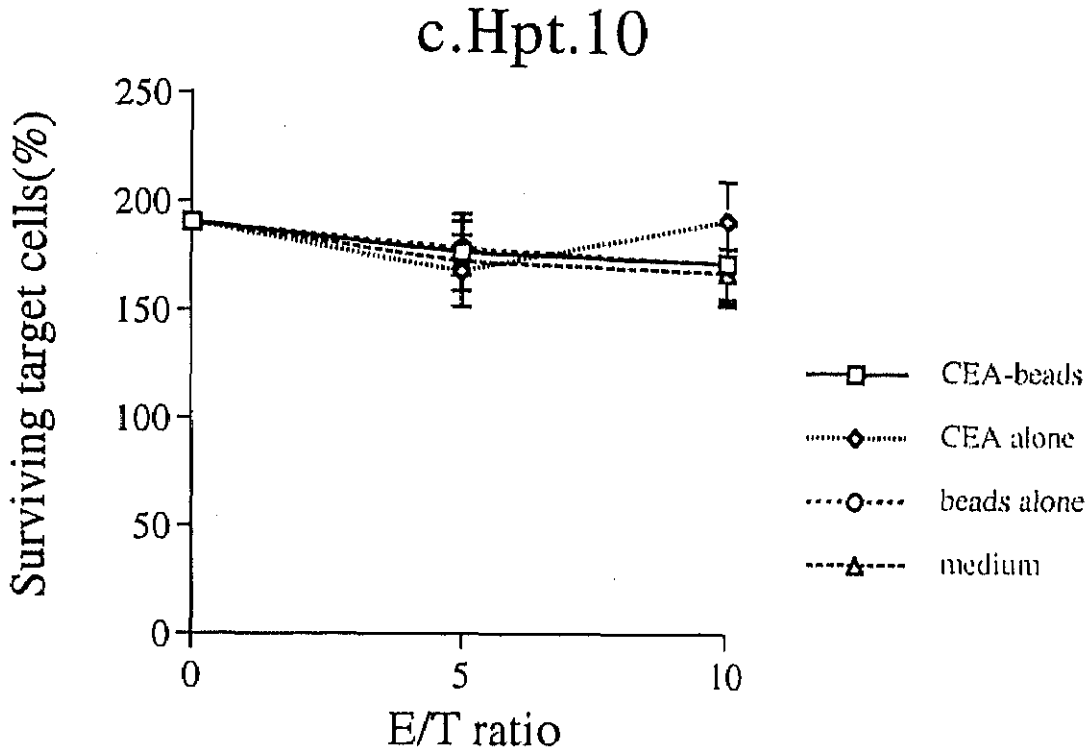


Fig. 8c. Cytotoxicity of the effector cells against Hpt. 10, CEA-negative HLA-A2402 renal carcinoma cells. The killing assay was performed by CV assay as described in Materials and Methods. Each point reoresents the mean of four replicates and it accompanied by an error bar indicating SD.

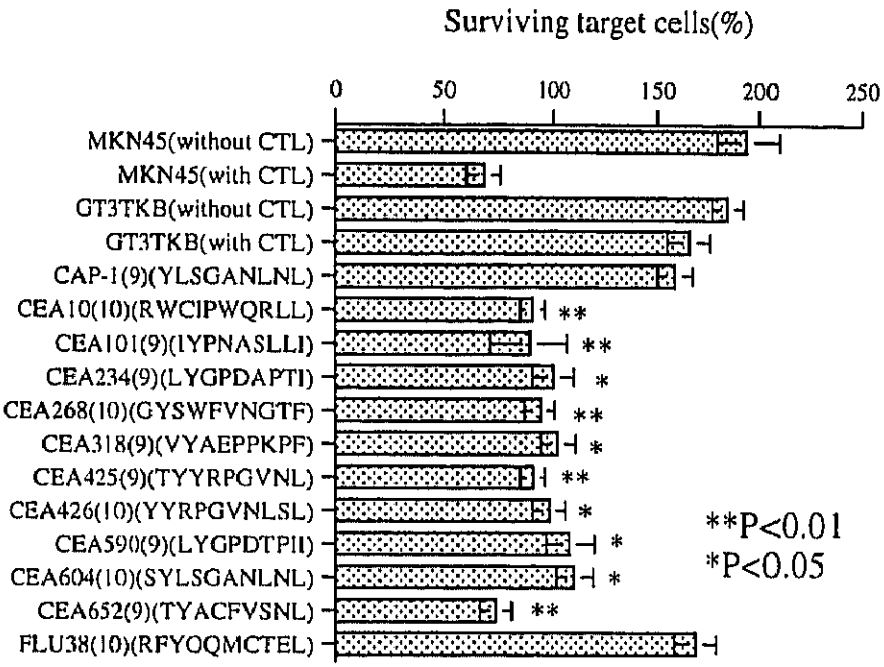


Fig. 9. Cytotoxicity of cytotoxic T lymphocytes (CTL) against CEA-peptide-pulsed cancer cells. GT3TKB cells carrying HLA-A2402 were incubated in the presence of an HLA-A2402-binding peptide derived from CEA at a final concentration of 50 ug/ml for 1 hr and submitted to the killing assay with the CTL. Each peptide sequence is shown in the parentheses from the fifth line onwards. The killing assays were carried out at an E/T ratio of 10 as described in Materials and Methods. Each bar represents the mean of four replicates and it accompanied by an error bar indicating SD.

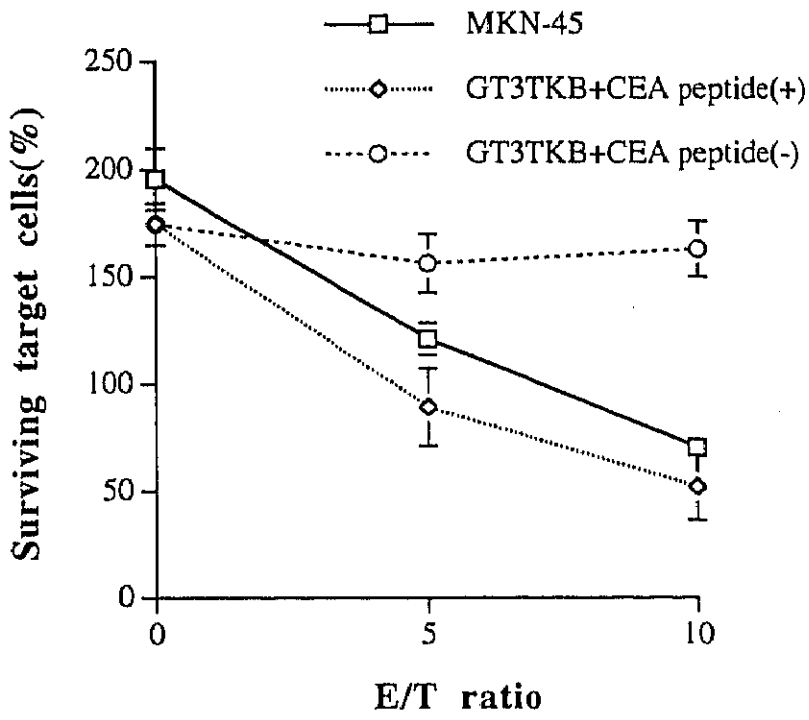


Fig. 10. Killing activity of the CTL generated on CEA652(9)-pulsed and fixed adherent cells from an HLA-A2402 donor. Cytotoxicity of the CTL generated was assayed by the CV staining method (see Materials and Methods). Each bar represents the mean of four replicates and it accompanied by an error bar indicating SD.

Table 5. Surface phenotype analysis of effector cells

Effector cells cultured with	Percentage positive cells		
	CD3	CD4	CD8
CEA-beads	98.3	2.2	**72.9**
CEA protein alone	98.4	11.6	29.9
Beads alone	96.7	28.4	25.6
Medium	98.4	11.8	30.2

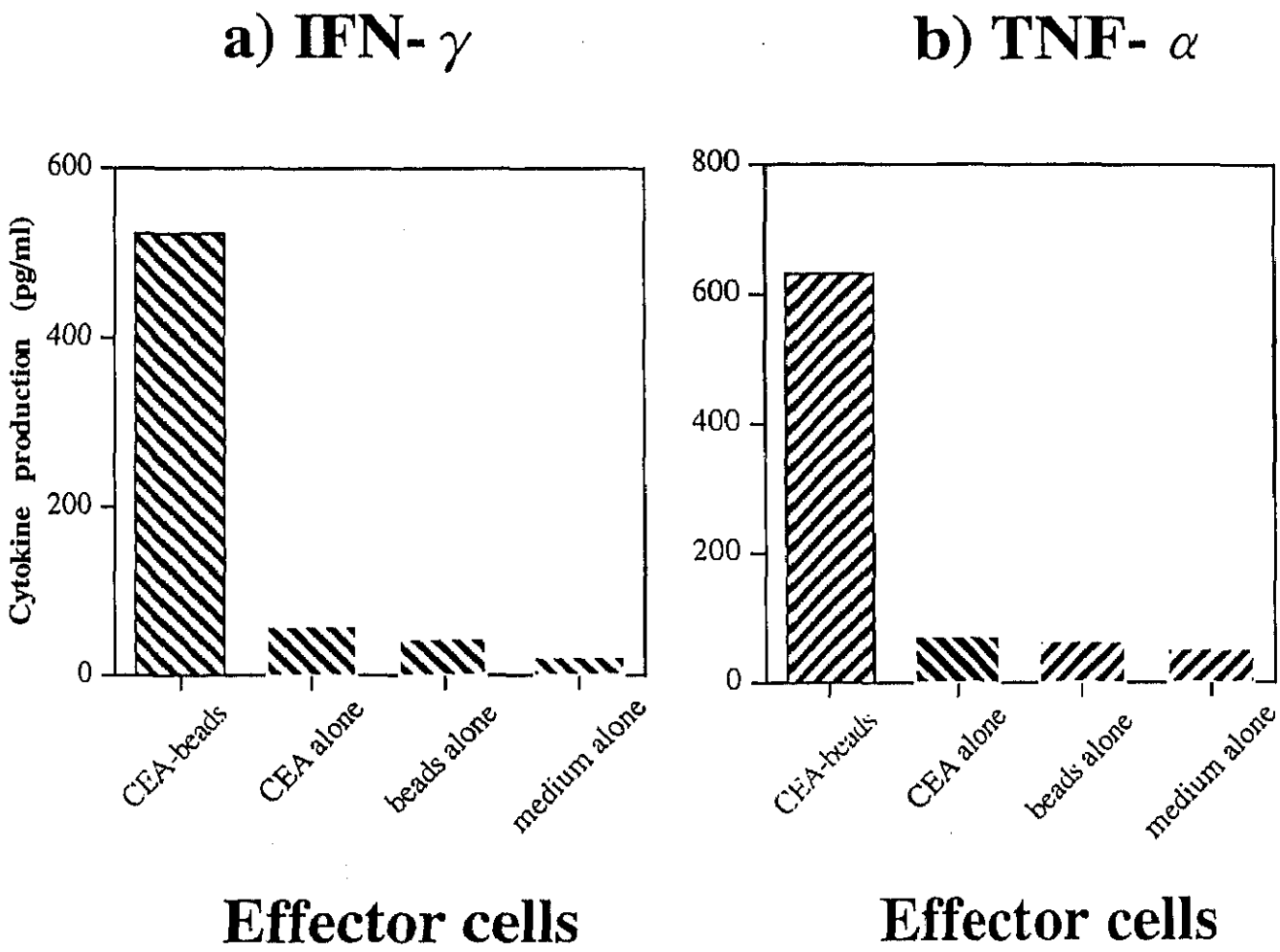


Fig. 11. Specific release of cytokine by CTL in response to CEA-beads pulsed APC

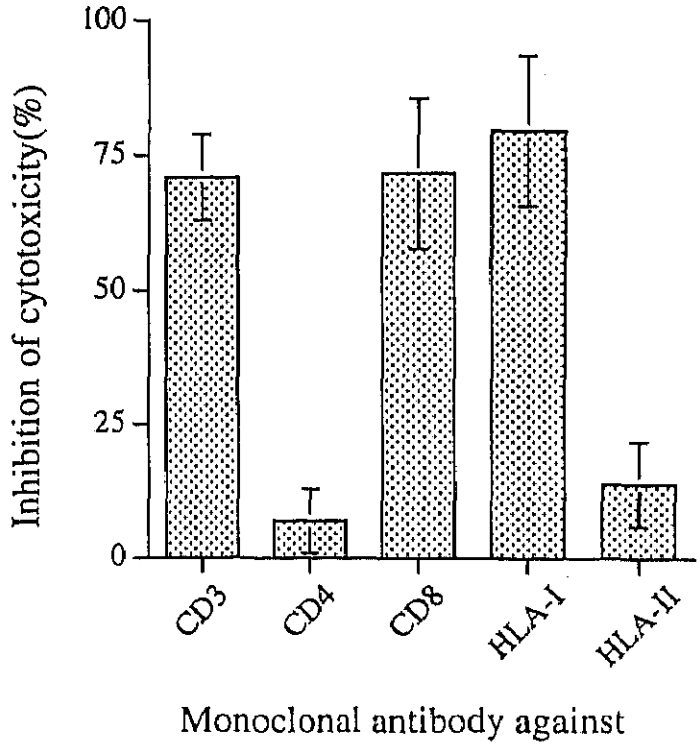


Fig.12. Inhibition of cytotoxicity of the CTL with monoclonal antibodies. Inhibition assays were carried out at an E/T ratio of 10 as described in Materials and Methods. Each point reoresents the mean of four replicates and it accompanied by an error bar indicating SD.

Discussion

The present results suggest that the cultured lymphocytes on the fixed adherent blood cells pre-loaded with CEA-beads contain MHC class I-restricted $CD8^+$ CTLs (Fig. 12) specific to CEA-producing carcinoma cells (Fig. 8a), but not, or at very low extent, to CEA-non-producing HLA-A2402 carcinoma cells (Fig. 8b and 8c). MHC- class II molecules were not involved in the cytotoxic response of the CTLs (Fig. 12). The effector cells cultured on the fixed adherent cells pre-loaded with CEA protein alone showed no specific cytotoxicity on any target cells (Fig. 8a, 8b and 8c). These observations provide evidence that the exogenous antigen on the latex beads was processed and presented by MHC-class I molecules after delivery into cells [108]. We could not detect significant killing activities in the lymphocyte population against K562 cells and Daudi cells, suggesting that the induced lymphocytes may have scarcely contain natural killer cells and lymphokine-activated killer cells.

We also demonstrated here that the CTLs recognized efficiently the CEA epitope peptide TYACFVSNL (CEA652(9)) and less efficiently other peptides so far tested (Fig. 9). CEA652(9) contains two anchor-motif amino acid residues, Y and L with the space of 6 amino acid residues, but the binding affinity to the HLA-A2402 molecule was

the third in the tested 11 peptides [K. Takesako and I. Nukaya, personal communication]. To our surprise, the peptide CEA10(10) (RWCIPWQRLL) that has only one anchor-motif amino acid residue L revealed a considerable antigenic activity to the CTLs. This amino acid sequence should not be included in the mature CEA protein. Presumably the purchased CEA preparation which has been extracted from a metastasis of human colon cancer contained the immature CEA protein. Although the HLA-A2 binding peptide, CAP-1, also shares one anchor-motif amino acid residue L [123], the CTLs exhibited no killing activity against the CAP-1 pulsed target cells (Fig. 9). This finding suggest that the CTLs are strictly restricted by the HLA-A24 molecules. It has been evidenced that phagocytic processing of exogenous antigens occurs in macrophages in which the processed antigenic peptides bind to MHC-class I molecules [138]. Previous studies have shown that latex beads-bound OVA can be processed for presentation by MHC-class I molecules and can generate CTL response both in vitro and in vivo [119,139]. We also assume that, in the present experiments, macrophages have played essential role at the CEA processing after phagocytosis of the CEA-beads. Although it is well known that induction of CTL response is strongly primed by the potent antigen presenting cells, dendritic cells [140,141], which act complementarily with macrophages [142], we are not able to determine at present how

much the non- or poorly-phagocytic dendritic cells have contributed in the present CTL induction.

Since the present CTL induction was carried out strictly under autologous condition as the lymphocytes were cultured for weeks on the autologous adherent blood cells that previously phagocytized CEA-latex beads and then were fixed with formalin, the results strongly imply that the technique described here will be able to induce autologous CTLs of the tumor-bearing patients against the CEA producing autologous cancer cells in vitro. Although at present we do not have the chance to test the cytotoxic activity of CEA-specific CTLs against the autologous CEA-producing cancer cell lines, Alters et al. [143] showed that dendritic cells pulsed with the HLA-A2 restricted CEA peptide CAP-1 (YLSGANLNL) can generate a CEA specific CTL response in vitro from both unimmunized carcinoma patients and healthy donors.

We have selected HLA-A24 carriers as the source of lymphocytes since they share approximately 60% of Japanese population. As the case of HLA-A2 [143], it is possible to induce HLA-A24 restricted CTL with 9-mer or 10-mer peptides derived from CEA that bind to the HLA-A24 molecule [Nukaya et al., personal communication]. We also demonstrated that loading of the CEA peptide to the adherent cells resulted in the generation of the CTLs in vitro (Fig. 10). By this approach, however, so many trials must be made to identify the real

epitope peptide among the candidate peptides. Our present approach is simple since CEA whole protein was used as the antigen and previous selective culture of dendritic cells that are hard to proliferate was not required. Fixed antigens on the adherent PBMC will provide long lasting stimulating effect on the expansion of CTLs [127]. Consequently, however, the CTLs responded polyclonally to the CEA epitope peptides (Fig. 9). To test the possiblity that CTL may have killed target cells by secreting cytokines such as IFN-g and TNF-a, we added their culture supernatant to the target cells. However, no killing of MKN-45 cells was observed after the 24 hr incubation (data not shown).

Since normal colorectal cells and many of epithelial cells in adults produce constantly a limited amount of CEA, one may hesitate to apply the CEA-specific CTLs for the treatment of CEA-producing carcinoma cells because of the possible occurrence of autoimmune disease caused by the CTLs. Kang et al. [128] reported that, in melanoma patients, the autologous tumor infiltrating lymphocytes (TILs) recognized the specific antigenic peptides of tyrosinase, a common enzyme in normal melanocytes. Practically, however, the patient showed a dramatic response without severe side effects to the adoptive immunotherapy with the autologous TILs that were used to identify the tyrosinase gene [69]. Therefore, we consider that CEA-specific CTLs are one of

feasible choices for the treatment of the patient who is suffering from multiple metastasis of the cancer producing high level of CEA. We also consider that, if any specific tumor antigens are available and their epitope peptides are unknown, the technique described here will be useful for the induction of autologous CTLs specific to the target tumor antigen even if the target cancer cells are not available.