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Host-oriented Peptide Evaluation Using Whole Blood Assay for Generating Antigen-specific Cytotoxic T Lymphocytes

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Abstract. *A whole blood assay using antigenic peptide was established to predict host cytotoxic T lymphocyte (CTL) precursor status. Blood samples from HLA-A24 donors and colorectal cancer patients were directly diluted with RPMI-1640 medium to a 20% blood concentration, then distributed to tubes and a peptide of an HLA-A24-restricted CEA peptide panel (20 μM) was added to the tubes. Incubation was performed for 4-5 days and supernatants were subjected to ELISA specific for IFN-gamma protein. It was observed that certain CEA peptides could stimulate the diluted blood samples to produce IFN-gamma. Only the peripheral blood mononuclear cells (PBMCs) that were purified from the IFN-gamma-positive samples of the whole blood assay showed positive spots, detected with IFN-gamma ELISPOT assay, and could proliferate with the stimulation of immobilized anti-CD3 antibody plus interleukin-2 (CD3/IL-2 system). The proliferating PBMCs expressed cytotoxic activity against HLA-A24+ CEA-expressing tumor cells and the TISI target cells pulsed with the CEA peptide that had been used to stimulate the PBMCs to produce IFN-gamma, but they did not kill the target cells pulsed with peptides that had failed to stimulate IFN-gamma production, nor did they kill the target cells alone. Theses findings suggest that the IFN-gamma production of the blood samples detected by the whole blood assay identifies the peptide that can induce the CEA antigen-specific CTL response. Detection of IFN-gamma gene expression using real-time-PCR analysis could identify the peptide within 6 hours, which is earlier than the protein analysis by ELISA. The whole blood assay using the CEA peptide panel for healthy donors and colorectal cancer patients revealed that IFN-gamma-inducible peptides were different among the individual samples tested, indicating that the CEA peptides that should be used for generating CTLs are different in individual patients. The whole blood assay using a CEA antigen peptide panel is simple and beneficial for identifying candidate peptides. The host-oriented peptide evaluation (HOPE) approach may provide hope for the augmentation of clinical efficacies for peptide-based cancer immunotherapy.*

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The discovery and molecular cloning of the crucial lymphocyte growth factor, interleukin-2 (IL-2), has facilitated the clinical application of the adoptive immunotherapy (AIT) of cancer using autologous lymphocytes activated *in vitro* with IL-2 (1). Disease-associated immunosuppression in patients with cancer can disturb the effective emergence of anti-tumor responses *in vivo* (2), so that the adoptive transfer of effector lymphocytes, after being educated and activated *ex vivo* to recognize tumor cells, would, theoretically, be an effective treatment for cancer. Among the techniques developed, the use of lymphokine-activated killer (LAK) cells (3), autolymphocyte therapy (ALT) (4) and tumor-infiltrating lymphocytes (TIL) (5) have been the best studied. While further trials are ongoing, thus far these approaches have not consistently shown benefits in comparison to standard immune-based treatment with biologic response modifiers (6). The success of adoptive cellular therapy depends on the ability to select optimally or produce cells genetically with the desired antigenic specificity, and then induce cellular proliferation while preserving the effector function, engraftment and homing abilities of the lymphocytes. Unfortunately, many previous clinical trials were carried out with adoptively transferred cells that were propagated in what are now understood to be sub-optimal conditions that impair the essential functions of the adoptively transferred cells (7).

The molecular understanding of antigen presentation and recognition has permitted us to establish new approaches to AIT for metastatic cancer patients, among which the use of dendritic cells (DCs) is encouraging (6). We have previously

Table I. *Peptides used in this study.*

CEA10(10)	RWCIPWQRLL
CEA101(9)	IYPNASLLI
CEA234(9)	LYGPDAPTI
CEA268(10)	QYSWFVNGTF
CEA318(9)	VYAEPPKPF
CEA425(9)	TYYRPGVNL
CEA426(10)	YYRPGVNLSL
CEA590(9)	LYGPDTPII
CEA604(10)	SYLSGANLNL
CEA652(9)	TYACFVSNL

published a novel system for generating cytotoxic effector lymphocytes using DCs, the nomenclature of which is "peptide-pulsed DC-activated killer" (PDAK) cells (8). It has been shown that an antigenic protein has several antigenic epitopes presented on host HLA molecules (9, 10), so that we should choose, if possible, the best peptide sequence for stimulating patients' lymphocytes to be effective PDAK cells. This may depend on the patients' precursor status of cytotoxic T lymphocytes (CTLs) reactive with the peptide. In this paper, we tried to establish a screening method for defining the candidate peptides appropriate for generating PDAK cells in individual patients.

Materials and Methods

Cells. A human B-lymphoblastoid cell line, TISI cells, that is defective in antigen processing but expresses the HLA-A24 allele and can effectively present exogenously supplied peptides, was used (11). The gastric cancer cell line MKN45 (12) expresses HLA-A24 and carcinoembryonic antigen (CEA). Both cell lines were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS).

Whole blood assay. Peripheral venous blood was collected by venipuncture from HLA-A24+ healthy donors (n=5) and patients with untreated colorectal cancer (n=9) and then immediately heparinized. The heparinized venous blood was diluted with RPMI-1640 medium (Invitrogen Corp., Carlsbad, CA, USA), 1 ml of which was distributed to polypropylene round-bottom tubes (Becton, Dickinson & Co., Franklin Lakes, NJ, USA). One antigenic peptide of a peptide panel was added to one tube. The panel of HLA-A24-restricted CEA peptides (TAKARA BIO INC., Ohtsu, Japan) used in this study is shown in Table I. Flu38 was prepared as a positive control and HIV (SIGMA Genosys, Ishikari, Japan) was used as a negative control. Incubation was performed at 37˚C in a humidified 5% CO_2 incubator. Samples were resuspended and centrifuged and supernatants were collected and stored at –30˚C until used. We measured IFN-gamma protein in the supernatants with enzyme-linked immunosorbent assay (ELISA) using the Quantikine Human IFN-gamma immunoassay kit (R&D Systems Inc., Minneapolis, MN, USA) according to the manufacturer's instructions. The density of each well was detected as absorbance at 450 nm with the correction wavelength set at 540nm in the Emax™ Microplate Reader (Molecular Devices Corporation, Sunnyvale, CA, USA). In preliminary experiments, the optical density at 630 nm of the supernatant was also measured to determine hemolytic changes of the blood culture (13).

ELISPOT assay. IFN-gamma-secreting cells were analyzed with enzyme-linked immunospot (ELISPOT) assay. Peripheral blood mononuclear cells (PBMCs) were isolated from the samples of the whole blood assay mentioned above by standard density gradient centrifugation using Lymphopep (Axis-Shield PoC AS, Oslo, Norway). The ELISPOT assay was conducted on cells (4 x 10^4/well) using Human IFN-gamma ELISpot Kit (R&D Systems Inc., Minneapolis, MN, USA) according to the manufacturer's instructions.

Proliferation assay. PBMCs (5 x 10^5 /ml) isolated after the whole blood assay were resuspended in RPMI-1640 medium containing 40 IU/ml IL-2 and 2% autologous serum (complete medium) and stimulated in a 24-well tissue culture plate, which had been coated with 1 μg/ml anti-CD3 antibody (OKT-3, Kyowahakko, Tokyo, Japan) for more than 24 hours and rinsed with RPMI-1640 medium more than 3 times (14). The cells were incubated at 37˚C in a humidified CO_2 incubator for 7 to 14 days with the medium half-changed with fresh complete medium twice a week. Cells activated with IL-2 plus anti-CD3 antibody stimulation (CD3/IL-2 system) were washed, resuspended in the medium, distributed in a 96-well microtiter plate in triplicate (100 μl/well) and pulsed with 1 μ Ci ^{3}H-thymidine, followed by another incubation for 8 hours. The incubation was terminated by harvesting the cells and their radioactivities were counted with a scintillation counter (Packard, USA).

Cytotoxicity assay. The cytotoxic activity of lymphocytes activated with peptides and the CD3/IL-2 system was determined by a standard ^{51}Cr-releasing assay. First, target cells were pulsed with peptide and labeled with ^{51}Cr. Target cells and effector lymphocytes were cocultured in 96-well round-bottomed microtiter plates in triplicate at effector-to-target (E/T) ratios of 10 and 20 in a volume of 200 μl. After a 4-hour incubation, the radioactivity of the supernatants was measured using an auto-gamma scintillation counter (Packard). Spontaneous release was determined in wells containing the target cells alone and maximum release was obtained by adding 100 μl of 1% Triton X-100 solution to the target cells instead of the effector cells. Cytotoxic activity was calculated from triplicate samples by the following formula: cytotoxic activity (per cent) = (experimental release [cpm] - spontaneous release [cpm])/(maximal release [cpm] - spontaneous release [cpm]) X 100.

Real-time quantitative PCR with the LightCycler™. Total RNA was extracted from PBMC isolated from samples of whole blood assay according to the protocol of the RNeasy Mini Kit (Qiagen, Wien, Austria). The reverse transcription reaction was carried out according to the protocol of Ready-To-Go You-Prime First-Strand Beads (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA). The LightCycler™-Primer Set IFN-gamma and LightCycler™-

Table II. *Optimal conditions for the whole blood assay using antigenic peptide.*

Blood concentration	Days in culture			
(%)	2	3	4	5
10	73[a]	102	113	155
	0.01[b]	0.02	0.03	0.02
20	192	237	303	298
	0.02	0.03	0.03	0.03
30	36	45	52	48
	0.04	0.05	0.07	0.09
40	38	72	64	60
	0.05	0.07	0.15	0.16
50	48	33	47	25
	0.08	0.12	0.18	0.38

Whole blood was diluted with medium and cultured for 2-5 days as indicated in the presence of 20 μM CEA652; the supernatant was subjected to ELISA for IFN-gamma (a) and measurement of optical density at 630 nm (b).

Table III. *Comparison among IFN-gamma production, IFN-gamma spots and lymphocyte proliferative responses.*

	Pt-1			Pt-2		
Peptide	IFN-g	Spots	^{3}H-TdR	IFN-g	Spots	^{3}H-TdR
None	0	0	104	0	0	331
FLU38(10)	100	12	4645*	64	14	655*
CEA10(10)	0	0	98	0	-	-
CEA101(9)	0	0	88	0	0	294
CEA234(9)	0	0	101	725	133	8331*
CEA268(10)	0	0	99	0	0	306
CEA318(9)	0	0	109	0	-	-
CEA425(9)	0	0	87	470	77	3350*
CEA426(10)	0	0	105	0	-	-
CEA590(9)	130	16	623*	0	-	-
CEA604(10)	0	0	87	540	104	6744*
CEA652(9)	640	72	2036*	240	48	2087*

Blood samples were diluted with medium and stimulated with peptides, then the supernatants were subjected to ELISA specific for IFN-gamma protein. PBMCs were harvested from the same blood samples after peptide stimulation and subjected to IFN-gamma ELISPOT analysis. Harvested PBMCs were stimulated with the CD3/IL-2 system and ^{3}H-TdR uptakes were determined. Standard deviations of ^{3}H-TdR uptakes were less than 15% of each value. Significant differences from the value with no peptide, $*p<0.05$. Pt, patient.

Primer Set G6PDH (Search GmbH, Heidelberg, Germany) were used in the analysis of IFN-gamma and G6PDH gene expression. The PCR reactions were performed in a LightCycler™ instrument using LightCycler™ FastStart DNA Master SYBR Green I (Roche Diagnostics GmbH, Mannheim, Germany). Thermocycling was done in a final volume of 20 μl containing 10 μl of DNA sample (diluted 1:10) and other components; 2 μl of LightCycler™ Primer mix; 2 μl of LightCycler™ FastStart DNA Master SybrGreen I (including Taq DNA polymerase, reaction buffer, deoxynucleotide triphosphate mix, SYBR Green I dye and 10mM $MgCl_2$); and 6 μl of PCR grade water. After 10 minutes at 95˚C to denature the cDNA and to activate the Taq DNA polymerase, the cycling conditions were as follows: 35 cycles consisting of denaturation at 95˚C for 10 seconds, annealing at 68˚C for 10 seconds and extension at 72˚C for 16 seconds. After PCR, a melting curve was constructed by increasing the temperature from 58˚C to 95˚C with a temperature transition rate of 0.1˚C/second. IFN-gamma and G6PDH sequences were amplified in duplicate from the patient samples. The assay was completed in ~1 hour. To ensure that the correct product was amplified in the reaction, all samples were separated by 2% agarose gel electrophoresis. The LightCycler™ instrument measured the fluorescence of each sample in every cycle at the end of the annealing step. The Fit Points Method was used to determine the cross point (Cp) automatically for the individual samples. The LightCycler™ software constructed the calibration curve by plotting the Cp *vs* the logarithm of the number of copies for each calibrator. The numbers of copies in unknown samples were calculated by comparing their Cps with the calibration curve. To correct for differences in both RNA quality and quantity between samples, the data were normalized using the ratio of the target cDNA concentration to that of G6PDH.

Statistical analysis. Statistical analysis was conducted by a χ^2 test or Student's *t*-test using StatView software (Version 5) on a Macintosh computer.

Results

Optimal incubation period and blood concentration for whole blood assay. At first, in order to determine the optimal conditions for the incubation period and blood concentration for the whole blood assay that detects IFN-gamma production by peptide stimulation, blood samples from HLA-A24 donors were directly stimulated under various incubation conditions with a CEA652 peptide that has been reported to have the most potent motif for binding to HLA-A24 molecules (9) (Table II). IFN-gamma production in the supernatant was apparently detectable with ELISA in the tubes of 20% blood sample, even on day 2. IFN-gamma production increased thereafter and peaked on day 4, showing a maximal production of 303 pg/ml. The supernatant from the tube of the 5-day culture of the 20% blood sample showed a similar IFN-gamma production of 298 pg/ml. The supernatant from the tubes of 10% blood samples showed lower IFN-gamma production and concentrations of 30% or more blood

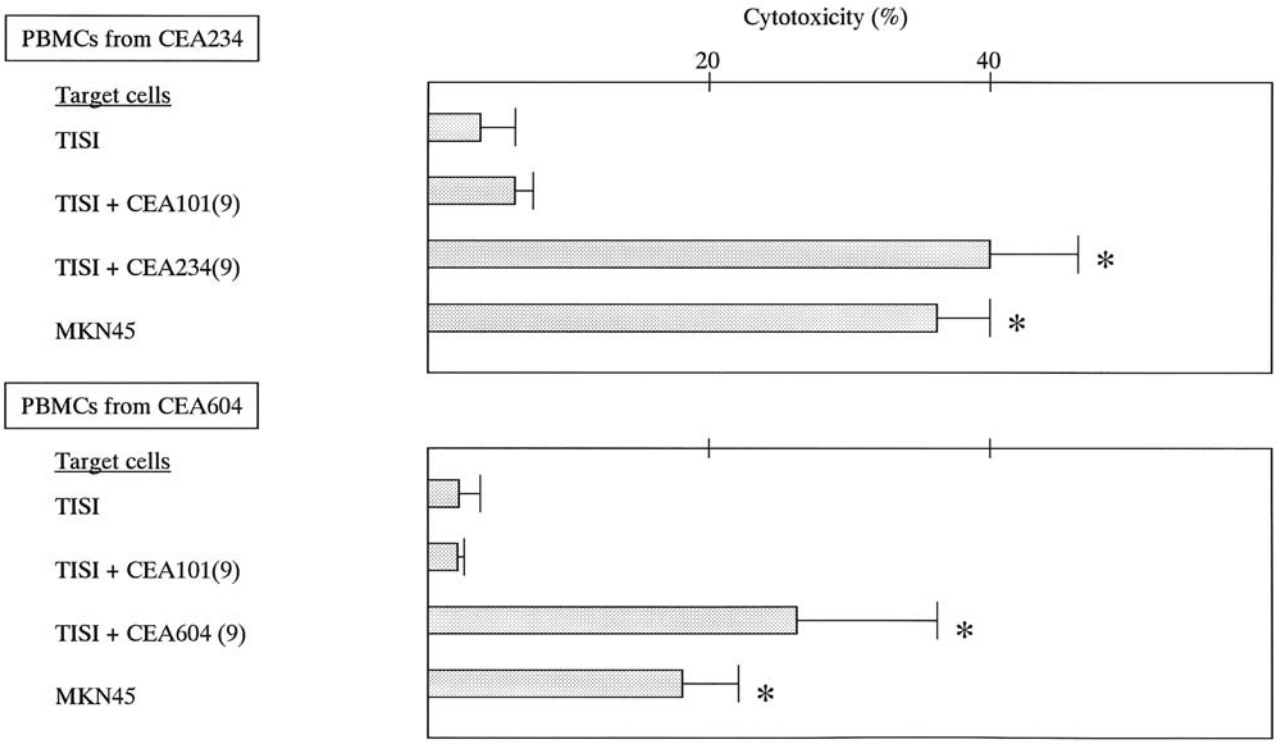

Figure 1. *Cytotoxic activity of PBMCs from IFN-gamma-positive whole blood assay. PBMCs were harvested from IFN-gamma-positive whole blood assay, stimulated with the CD3/IL-2 system and subjected to cytotoxicity assay using the target cells indicated. Significant differences from the cytotoxic activity against TISI cells alone, *p<0.01.*

Table IV. *Diversity of IFN-gamma-inducible peptides in colorectal cancer patients.*

Peptide	HD-1	HD-2	HD-3	HD-4	HD-5	Pt-1	Pt-2	Pt-3	Pt-4	Pt-5	Pt-6	Pt-7	Pt-8	Pt-9
None	125	0	0	50	0	-	-	-	0	-	0	0	0	0
FLU38(10)	150	285	170	100	220	-	-	-	0	-	500	285	100	85
CEA10(10)	325	210	0	0	120	0	0	0	0	67	0	20	55	0
CEA101(9)	75	35	100	0	0	0	75	0	0	90	200	250	0	0
CEA234(9)	0	170	50	80	70	0	20	0	725	0	0	40	0	170
CEA268(10)	75	80	0	525	0	0	0	0	0	140	80	40	0	0
CEA318(9)	240	0	0	60	0	106	0	20	0	31	1000	100	10	0
CEA425(9)	975	0	30	30	100	4547	0	0	470	126	120	20	10	0
CEA426(10)	550	65	60	40	0	0	0	0	0	0	30	20	0	70
CEA590(9)	300	320	0	0	0	0	0	0	0	0	0	60	180	925
CEA604(10)	0	50	0	50	40	227	0	25	540	0	300	50	10	0
CEA652(9)	255	560	670	230	280	0	140	339	240	216	275	350	640	0

Whole blood assay was performed in HLA-A24 healthy donors and colorectal cancer patients and IFN-gamma production was shown. HD, healthy donor; Pt, patient

samples had incomparable IFN-gamma production. At the same time, the optical density at 630 nm of the same culture supernatants was determined. The optical density at 630 nm increased in parallel with blood concentration and the sample with 50% concentration and a 5-day culture showed the highest optical density of 0.38. Collectively, we determined that the optimal conditions of the whole blood assay were 4-5 days for the incubation period and a 5-fold dilution.

ELISPOT assay for IFN-gamma-positive PBMCs. We next performed the ELISPOT assay on PBMCs collected from

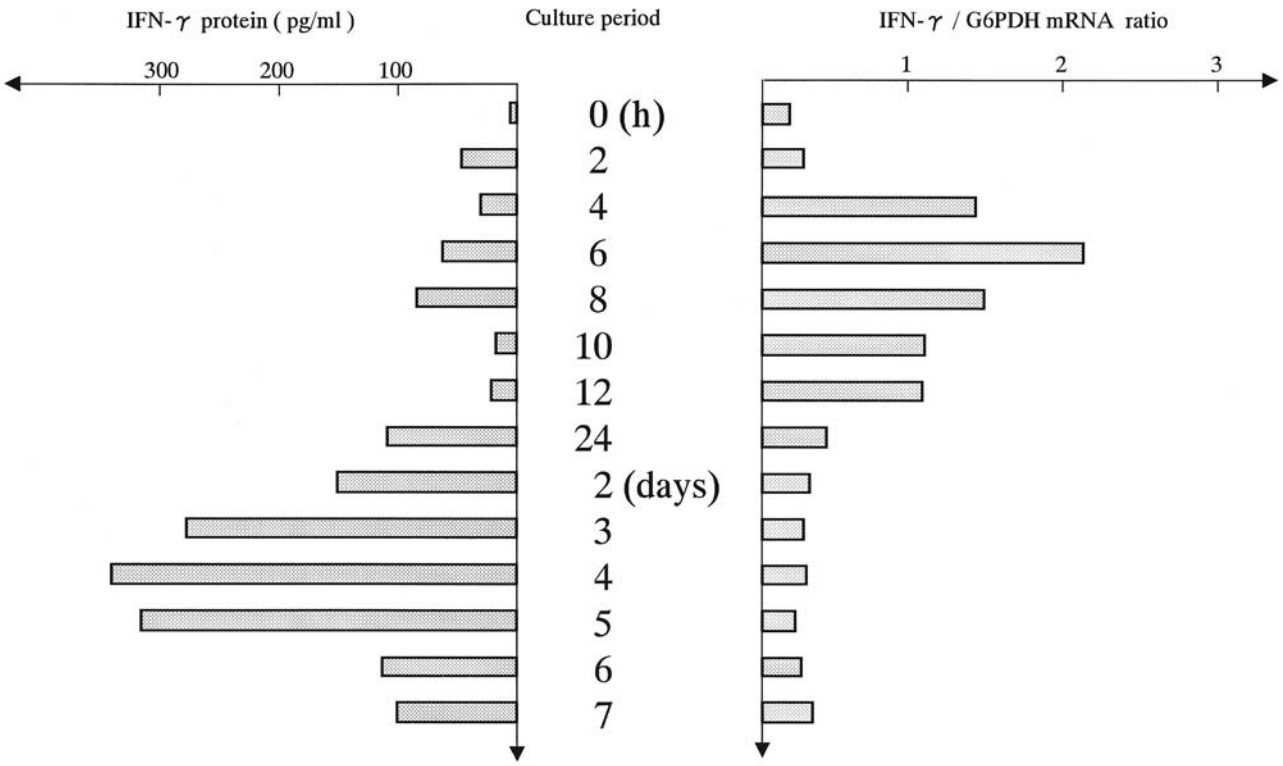

Figure 2. *Detection of IFN-gamma gene by real-time PCR. Whole blood assay was performed using CEA652 peptide and IFN-gamma expression was analyzed at various times by protein levels using ELISA and by mRNA levels using real-time-PCR.*

the whole blood assay stimulated with a CEA peptide panel. Representative data are shown in Table III. In Pt-1, CEA652 and CEA590 peptides were active in stimulating IFN-gamma production that was evaluated with the whole blood assay, which showed IFN-gamma productions of 640 and 130 pg/ml, respectively. The ELISPOT assay on the same PBMCs purified from the whole blood assay of Pt-1 showed positive spots only for the PBMCs purified from CEA652 and CEA590 peptide tubes, with these PBMCs showing 72 and 16 spots, respectively. The assay showed no spots for the PBMCs from the other tubes. Similar results were obtained in Pt-2, in which CEA234, 425, 604 and 652 were active in stimulating IFN-gamma production in the whole blood assay; PBMCs from the IFN-gamma-positive tubes could make IFN-gamma spots, but those from the other tubes could not.

Proliferative responses of IFN-gamma-positive PBMCs. PBMCs collected from the whole blood assay were stimulated with the CD3/IL-2 system and the proliferative responses were evaluated by ^{3}H-TdR uptakes (Table III). PBMCs from the CEA652 and CEA590 tubes of Pt-1 could proliferate by CD3/IL-2 stimulation, showing ^{3}H-TdR uptake of 2036 and 623 cpm, respectively. This ^{3}H-TdR uptake was significantly higher than those of PBMCs from IFN-gamma-negative tubes ($p<0.01$). Similar results were obtained in Pt-2. Only the PBMCs from the IFN-gamma-positive tubes could proliferate due to the CD3/IL-2 stimulation, but those from the other tubes could not.

Cytotoxic activity of PBMCs stimulated with the CD3/IL-2 system. In order to clarify whether or not the IFN-gamma production detected with the whole blood assay indicates truly antigenic peptide-specific immune responses, a cytotoxicity assay was developed for PBMCs purified from IFN-gamma-positive tubes in the whole blood assay (Figure 1). The PBMCs that were collected from the CEA234 tube of Pt-2 and stimulated with the CD3/IL-2 system did not kill TISI target cells alone. The PBMCs also did not kill TISI cells pulsed with the CEA101 peptide that could not stimulate IFN-gamma production for Pt-2 PBMCs. The PBMCs did kill TISI target cells pulsed with CEA234, showing a cytotoxic activity of 40% at an E/T ratio of 10. Similar results were obtained in the PBMCs from the CEA604 tube of Pt-2. These PBMCs from CEA234 and 604 killed MKN45 target cells that express HLA-A24 and CEA molecules.

Host-oriented detection of IFN-gamma-inducible peptide with whole blood assay. The whole blood assay using the CEA peptide panel was performed to detect the IFN-gamma-inducible peptides in HLA-A24+ healthy donors and HLA-A24+ colorectal cancer patients (Table IV). The highest levels of IFN-gamma production in each blood sample were observed with stimulations of CEA425, 652, 652, 268 and 652 in healthy donors 1 to 5, respectively, and with stimulations of CEA425, 652, 652, 234, 652, 318, 652, 652 and 590 in colorectal cancer patients 1 to 9, respectively. These levels ranged from 280 to 975 pg/ml in the healthy donors and from 140 to 4547 pg/ml in the patients.

Use of real-time PCR for detecting IFN-gamma-inducible peptide in whole blood assay. The IFN-gamma effects on the peptide stimulation in the whole blood assay were analyzed at various times at the mRNA level using real-time PCR in parallel with analyses performed at the protein level by ELISA (Figure 2). The production of IFN-gamma protein by stimulation with CEA652 peptide was detectable by ELISA on day 2, peaked on day 4, then decreased. The IFN-gamma gene expression was detectable only 4 hours after the stimulation and it peaked at 6 hours after the stimulation, then decreased. The IFN-gamma gene expression was no longer detectable on the days after the stimulation.

Discussion

Cytokine production profiles have been studied in relation to patients' immunity and are, in general, measured with purified PBMCs (15). However, it has been reported that a good correlation is obtained on comparing PBMC cultures with the whole blood system if the cell number is taken into account, and that whole blood culture is a simple and reproducible method for the measurement of mitogen-induced cytokine production (16). Our previous study demonstrated that the clinical responses of locoregional immunotherapy for malignant effusion were correlated with cytokine profiles analyzed using whole blood and whole effusion assay (17). In this study, CEA peptides could stimulate IFN-gamma production in the whole blood assay. This significance, however, must be clarified on a scientific basis.

First, the culture conditions of the whole blood assay must be established. Our experiments showed that the optimal conditions for the whole blood assay were an incubation period of 4-5 days and a 5-fold dilution. Although a more concentrated blood sample would have contained more CTL precursor that was able to produce IFN-gamma by CEA peptide stimulation, a greater concentration of the blood sample did not always produce more IFN-gamma. The dilution of 20% was optimal for preparing the samples for IFN-gamma measurement. The optical density at 630 nm increased with greater blood concentration, indicating that a more concentrated blood sample has increased hemolysis (13). The hemolysis may worsen the culture conditions and disturb the IFN-gamma production.

Second, the significance of the IFN-gamma production of the whole blood assay with CEA peptide stimulation should be clarified. IFN-gamma has been the key cytokine used in the monitoring of specific immune responses. For example, tumor-antigen encoding genes MAGE (18), MART-1/Melan-A (19) and SART (20) were discovered with the pairs of autologous tumor cells and their CTLs using IFN-gamma production as an indicator. However, did the IFN-gamma production in our study truly reflect peptide-specific responses? It was observed that only the PBMCs stimulated with CEA peptides to produce IFN-gamma could grow by means of stimulation of the CD3/IL-2 system. Moreover, these grown PBMCs killed HLA-matched CEA-expressing tumor cells and target cells pulsed with the peptide that had been used to stimulate the PBMCs, but did not kill target cells pulsed with the peptide that failed to stimulate IFN-gamma production and did not kill target cells alone. These findings indicate that activated PBMCs from the IFN-gamma-positive tubes of the whole blood assay express killing activity in an antigen peptide-specific manner. These results suggest that the IFN-gamma production that we observed in the whole blood assay directly indicates the peptide-induced specific immune responses. We would like to propose that the whole blood assay is a simple and beneficial method for screening the candidate peptides that can generate the antigen peptide-specific CTLs.

Based on the above results, the whole blood assay using the CEA peptide panel was performed in HLA-A24+ healthy donors and in patients with colorectal cancer. Surprisingly, it was observed that the IFN-gamma-inducible CEA peptides were different among the healthy donors and patients tested. Although CEA652 has been shown to have the most potent binding affinity for the HLA-A24 molecule and to induce CEA-reactive CTLs (9), it did not always stimulate sufficient IFN-gamma production of PBMCs in our blood samples. Previously, many vaccine studies for cancer treatment were designed using peptides that had a potent affinity to HLA molecules, but these studies have been unable to establish the expected clinical tumor responses (21, 22). Recently, the evidence-based vaccination protocol, in which notable tumor responses were generated, was published. In that study, the researchers chose only peptides that could stimulate PBMCs to produce IFN-gamma (23), indicating that the peptides to be used in cancer immunotherapy should be selected according not only to the HLA binding affinity of the peptide, but also to

the patients' CTL precursor status. It is reported that inappropriate peptides induce the affinity maturation of inappropriate T-cell receptors, which can disturb the appropriate peptide signalings for generating CTL responses (24), possibly resulting in the failure of cancer immunotherapy using antigenic peptides. This host-oriented peptide evaluation (HOPE) approach to augment tumor responses in the peptide-based clinical trials deserves a good deal of attention.

The time required for detecting candidate peptides to generate CTLs is another important issue in the immunotherapy of advanced cancer. It was reported that it took 14 days to detect candidate peptides to generate CTLs (10, 23). In our whole blood assay, it took 4-5 days, which suggests that our method may be more convenient for screening the candidate peptides. Finally, we attempted to establish a more rapid assay for predicting candidate peptides for generating CTLs by using the IFN-gamma gene (25). It was observed that the IFN-gamma response could be detectable within 6 hours using real-time-PCR instead of within 4-5 days using IFN-gamma-specific ELISA. This approach is more expensive and laborious than that of IFN-gamma-protein analysis, but can conserve the patients' limited time by permitting them to start peptide-based cancer immunotherapy earlier. Researchers who work in the treatment of advanced cancer patients should give the patients' time the highest consideration. The use of real-time-PCR for detecting the IFN-gamma gene may be a useful tool in the HOPE approach to peptide-based cancer immunotherapy.

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