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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 (4×10^4/well) using Human IFN-gamma ELISpot Kit (R&D Systems Inc., Minneapolis, MN, USA) according to the manufacturer's instructions.

Proliferation assay. PBMCs (5×10^5 /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

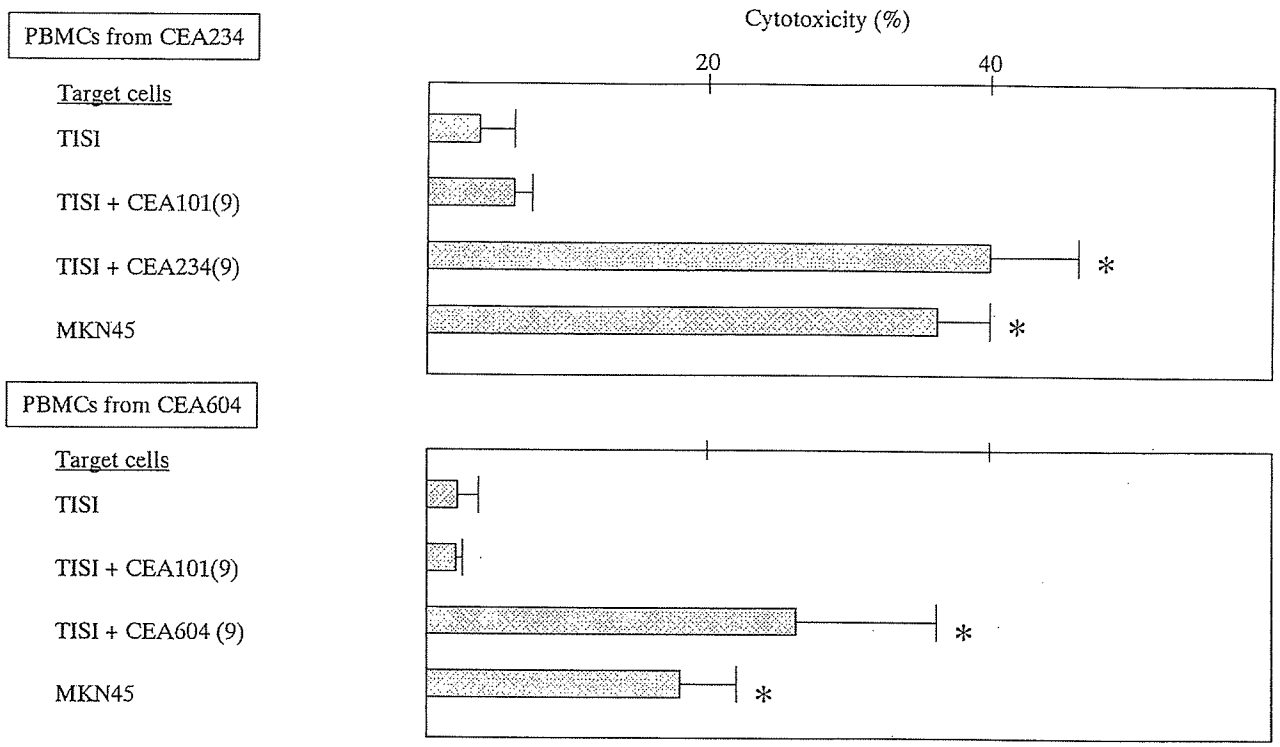

Figure 1. *Cytotoxic activity of PBMCs from IFN-gamma-positive whole blood assay. PBMCs were harvested from IFN-gamma-positive whole blood assay, stimulated with the CD3/IL-2 system and subjected to cytotoxicity assay using the target cells indicated. Significant differences from the cytotoxic activity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

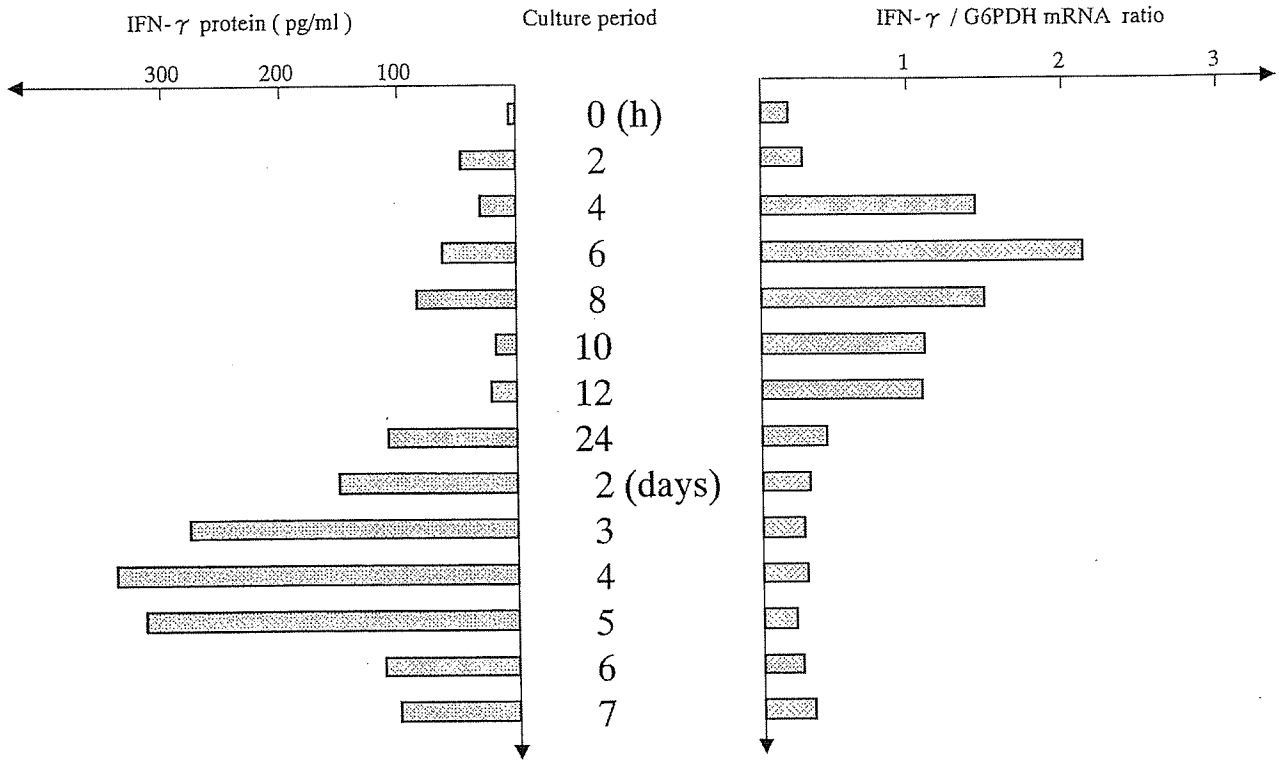

Figure 2. *Detection of IFN-gamma gene by real-time PCR. Whole blood assay was performed using CEA652 peptide and IFN-gamma expression was analyzed at various times by protein levels using ELISA and by mRNA levels using real-time-PCR.*

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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Postoperative immunosuppression cascade and immunotherapy using lymphokine-activated killer cells for patients with esophageal cancer: Possible application for compensatory anti-inflammatory response syndrome

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Abstract. Immunological parameters were measured in order to elucidate a postoperative immunosuppression mechanism in transthoracic esophagectomy for patients with esophageal cancer. Moreover, lymphokine-activated killer (LAK) cells were transferred just after the surgery to overcome the postoperative immunosuppression. Fifteen consecutive patients who underwent transthoracic esophagectomy were subjected to the postoperative measurement of immunological parameters. Ten patients who underwent open cholecystectomy served as controls. Heparinized venous blood was obtained pre- and postoperatively, and serum levels of cytokines IL-6 and IL-10 and immunosuppressive acidic protein (IAP) were measured. Peripheral blood lymphocytes were harvested and analyzed by flow cytometry for phenotype detection and by a mixed lymphocyte reaction for detecting concanavalin (Con)-A-induced or -non-induced suppressor activity. Another 29 consecutive patients who underwent transthoracic esophagectomy were randomly enrolled in a postoperative immunotherapy trial either with or without lymphokine-activated killer cells. It was found that, in the esophagectomy group, IL-6 and IL-10 increased postoperatively and peaked on day 1, followed by an increase in IAP, peaked again on day 4, with a profound decrease in helper and cytotoxic T-cell subsets, followed by increases in Con-A-induced (on day 7 or later) and spontaneous (on day 10) suppressor activities. These changes were minimal in the cholecystectomy group. LAK cell transfer restored the postoperative decrease in the helper and cytotoxic T-cell population, and there was a trend of reduction for postoperative remote infection such as pneumonia and surgical site infection in the LAK therapy group. Taken together, we would like to propose the existence of a postoperative immunosuppression cascade consisting of increases in cytokines and immunosuppressive proteins, decreases in helper and cytotoxic T-cell populations, and the development of suppressor T-cell activities in surgery for esophageal cancer. Postoperative adoptive transfer of LAK cells may be a novel clinical application in surgery for esophageal cancer as a means of treating this postoperative immunosuppressive condition that may be identical to the status of compensatory anti-inflammatory response syndrome (CARS).

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Abbreviations: AIT, adoptive immunotherapy; CARS, compensatory anti-inflammatory response syndrome; CD, cluster of differentiation; Con-A, concanavalin-A; FITC, fluorescein isothiocyanate; IAP, immunosuppressive acidic protein; IL, interleukin; LAK, lymphokine-activated killer; MLR, mixed lymphocyte reaction; PBMC, peripheral blood mononuclear cell; PE, phycoerythrin; SIRS, systemic inflammatory response syndrome; TIL, tumor-infiltrating lymphocyte; TNF, tumor necrosis factor; T-reg, regulatory T cell

Key words: postoperative immunosuppression, compensatory anti-inflammatory response syndrome, esophageal cancer, adoptive immunotherapy, lymphokine-activated killer cells

Introduction

Major thoraco-abdominal surgery, as represented by transthoracic esophagectomy, causes profound postoperative surgical stress, which can stimulate the production of pro-inflammatory cytokines, including TNF, IL-1 and IL-6, by immunocompetent cells (1). This condition, based on hypercytokinemia, was defined in 1991 through the consensus conference of the American College of Chest Physicians/Society of Critical Care Medicine (ACCP/SCCM), as systemic inflammatory response syndrome (SIRS) (2). On the other hand, an anti-inflammatory response is subsequently triggered by SIRS. This response is designated as compensatory anti-inflammatory response syndrome (CARS) (3). The anti-inflammatory cytokine IL-10 is a representative mediator in CARS status (4). Postoperative host responses are therefore understood as being a balance of SIRS and CARS, but they have not been

fully explored in surgery for esophageal cancer. Moreover, an imbalance between these dual immune responses, with an overwhelming release of pro- or anti-inflammatory cytokines, seems to be responsible for organ dysfunction and increased susceptibility to infections (5). Medical regulation of SIRS and CARS has been conducted to improve these adverse effects, but the most desirable means of regulation remains under investigation (6-11).

The discovery and molecular cloning of the crucial lymphocyte growth factor, interleukin-2 (IL-2), has facilitated the clinical application of adoptive immunotherapy (AIT) for cancer using autologous lymphocytes activated *in vitro* with IL-2 (12). We have carried out *ex vivo* cell therapy for cancer treatment using activated autologous lymphocytes, including lymphokine-activated killer (LAK) cells, tumor-infiltrating lymphocytes (TILs), and tumor-sensitized lymphocytes, but tumor responses are limited with regard to quality of life in locoregional administration for malignant effusion from gastrointestinal cancers (13). Ueda *et al* have reported an efficacy of AIT using LAK cells for metastatic esophageal cancer (14). The clinical trials using postoperative LAK cell transfer have demonstrated survival benefits in hepatocellular carcinoma (15) and lung cancer (16). However, the effects of AIT using LAK cells for postoperative stress status, SIRS and CARS have not yet been demonstrated.

In the present study, we explored the postoperative immunosuppression cascade in esophageal cancer surgery. Moreover, we conducted postoperative LAK therapy for patients with esophageal cancer and found that this therapy may be a possible therapeutic application for the postoperative immunosuppressive condition, CARS.

Materials and methods

Patients. Fifteen consecutive patients with histologically proven esophageal squamous cell carcinoma who underwent transthoracic esophagectomy were subjected to postoperative immunosuppression measurements, and 10 patients with open cholecystectomy served as controls. Another 29 consecutive patients with esophageal cancer were enrolled in the adjuvant LAK therapy study after sufficient written informed consent. The protocol was approved by the institutional review board. The patients were randomly assigned to either the standard therapy group or the LAK therapy group.

Collection of blood samples and lymphocytes. Heparinized venous blood was obtained from patients and healthy volunteers, and buffy coat and plasma were immediately separated by centrifugation (2,000 rpm, 30 min). The supernatant was subjected to measurements of cytokines and immunosuppressive acidic protein (IAP). The buffy coat was resuspended in RPMI-1640 medium, and the suspension was layered on Ficoll-Conray. Peripheral blood mononuclear cells (PBMCs) were isolated by gradient centrifugation (2,000 rpm, 30 min), washed twice, and resuspended in the medium containing 2% AB serum at a density of $1x10^6$/ml.

Cytokines and acute inflammatory proteins. Collected plasma samples were subjected to the measurement for IL-6, IL-10 and immunosuppressive acidic protein (IAP). IL-6 and IL-10 were measured using an ELISA kit according to the manufacturer's instructions (R&D Systems, Minneapolis, MN). IAP was measured by the SRL Co., Tokyo.

Flow cytometry. Fifty microliters each of the lymphocyte suspension ($5x10^5$) were stained with antibodies, washed, and then analyzed on a Cytoron (Ortho Diagnostic Systems, USA). The antibodies used were fluorescein isothiocyanate (FITC)-labeled anti-CD4 and -CD8 antibodies, phycoerythrin (PE)-labeled anti-CD45RA and -CD11b antibodies. All antibodies used were purchased from Becton-Dickinson, San Diego, CA.

Suppressor activity. A one-way mixed lymphocyte reaction (MLR) was performed as described in detail elsewhere (17). In brief, PBMCs were stimulated with 0 or 10 μg/ml concanavalin-A (Con-A, Boehringer, Germany) for 24 h at 37°C. Cells were treated with 50 μg/ml mitomycin-C for 1 h at 37°C, washed 3 times, and resuspended in the medium as effector cells ($5x10^5$/ml). Responder PBMCs ($5x10^5$/ml) were collected from healthy subjects. Effector and responder cells (1:1) were co-cultured in RPMI-1640 medium containing 2% AB serum for 4 days at 37°C in the presence of 15 μg/ml phytohemagglutinin (Difco, USA). Cells were pulsed with 5 μCi/ml ^{3}H-thymidine and incubated for another 8 h. Cells were harvested, and radioactivity was measured. Suppressor activity (SA) was calculated using the following formula: SA (%) = {1 - MLR (cpm)/responder cells alone (cpm)} x 100.

LAK cell generation and postoperative transfer. Plasmapheresis was performed using Haemonetics V30 (Haemonetics Corp., Braintree, MA) to harvest patients' white blood cells (WBCs) 2 weeks and 1 week before surgery. PBMCs were collected from WBCs by centrifugation. LAK cells were generated by culturing PBMCs (10^6/ml) for 2 weeks in RPMI-1640 medium containing 2% autologous serum and 400 U/ml IL-2 (Sionogi, Tokyo) supplemented with 2 mM l-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. The culture medium was half-changed with fresh medium containing IL-2 every 3-4 days. LAK cells ($1-3x10^9$ cells) were washed 3 times by saline, filtered through 200 μm mesh, resuspended in 100 ml saline, and administered intravenously twice a week (up to 6 times), postoperatively. Bacterial and endotoxin examinations were made 3 days before and on the day of administration.

Statistical analysis. Statistical analysis was conducted by the χ^2 test or Student's t-test using StatView software (Version 5) on a Macintosh computer. All values are presented as mean ± standard deviation, and $p<0.05$ was defined as statistically significant.

Results

Patients for postoperative measurements of immunological parameters. The esophagectomy (EG) group included 15 patients consisting of 11 men and 4 women, with a mean age of 58 years (Table I). In the cholecystectomy (CC) group, there were 3 men and 7 women whose mean age was 55. There was a significant difference between the EG and CC

Table I. Patients for postoperative measurements of immunological parameters.

Categories	Group	
	Cholecystectomy	Esophagectomy
Gender		
Male	3	11
Female	7	4
Age (mean ± SD)	55±11	58±10
Operation time (min, mean ± SD)	97±17	379±75
Blood loss (g, mean ± SD)	181±102	570±210

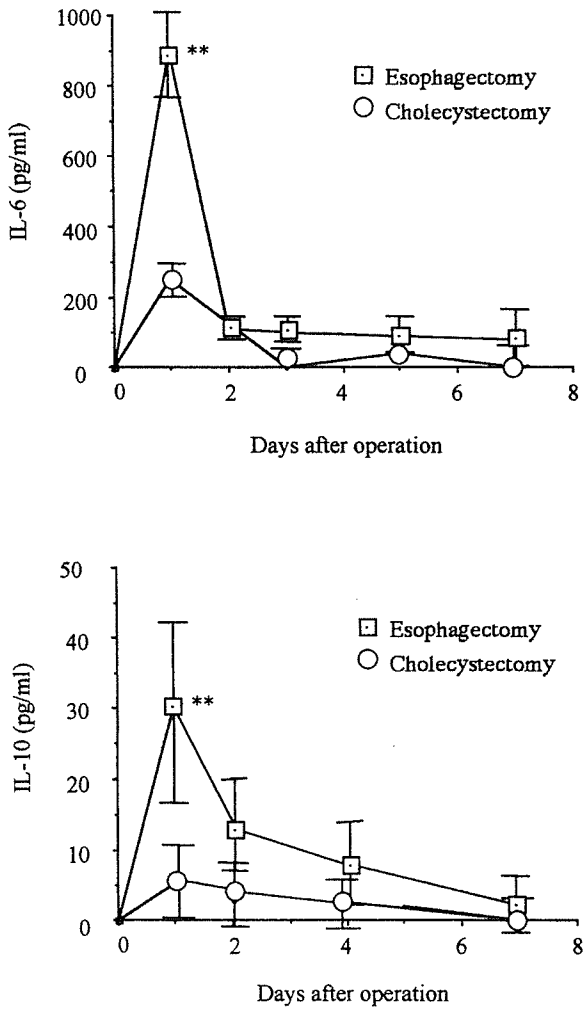

Figure 1. Postoperative changes in serum IL-6 and IL-10. Serum samples were postoperatively collected from patients who underwent transthoracic esophagectomy or open cholecystectomy, and IL-6 and IL-10 levels were measured. A significant difference, **p<0.01.

groups with regard to gender (p<0.05). Operative time and blood loss were 378 min and 570 g for the EG group, and 97 min and 181 g for the CC group, respectively. There were significant differences in these values (p<0.05).

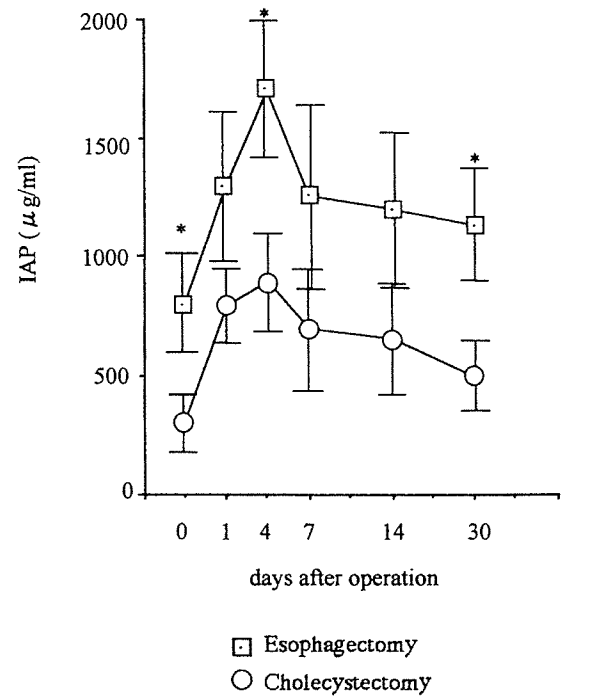

Figure 2. Postoperative changes in serum IAP. Serum samples were postoperatively collected from patients who underwent transthoracic esophagectomy or open cholecystectomy, and IAP levels were measured. Significant differences, *p<0.05.

Postoperative changes in serum IL-6 and IL-10 in patients with esophageal cancer. Postoperative cytokine levels were investigated (Fig. 1). Serum levels of IL-6 increased postoperatively, peaked on day 1, and then decreased. Serum levels of IL-10 fluctuated similarly with those of IL-6. Significant increases of IL-6 and IL-10 were observed on day 1 in the EG group compared with the CC group (p<0.01).

Postoperative changes in serum IAP. Postoperative changes in IAP, an acute-phase reactant protein, were investigated (Fig. 2). Serum levels of IAP increased postoperatively, peaked on day 4, and then decreased. Significant increases in IAP were observed before surgery, on day 4, and still on day 30 in the EG group compared with the CC group (p<0.05).

Postoperative changes in helper and cytotoxic T cell population. Postoperative changes in functional lymphocyte subsets, including the $CD4^+CD45RA^-$ helper subset and the $CD8^+CD11b^-$ cytotoxic T-cell subset, were investigated (Fig. 3). Changes in the helper and cytotoxic T-cell subsets were minimal in the CC group. In the EG group, however, levels of the helper subset significantly decreased compared with those in the CC group on day 1, continuing through day 14, and recovering, to a lesser extent, but still remaining at low levels on day 30 (p<0.05). The changes in the cytotoxic T-cell subset in the EG group were very similar to those of the helper subset.

Postoperative changes in suppressor activity. Con-A-induced and -non-induced (spontaneous) suppressor activities were

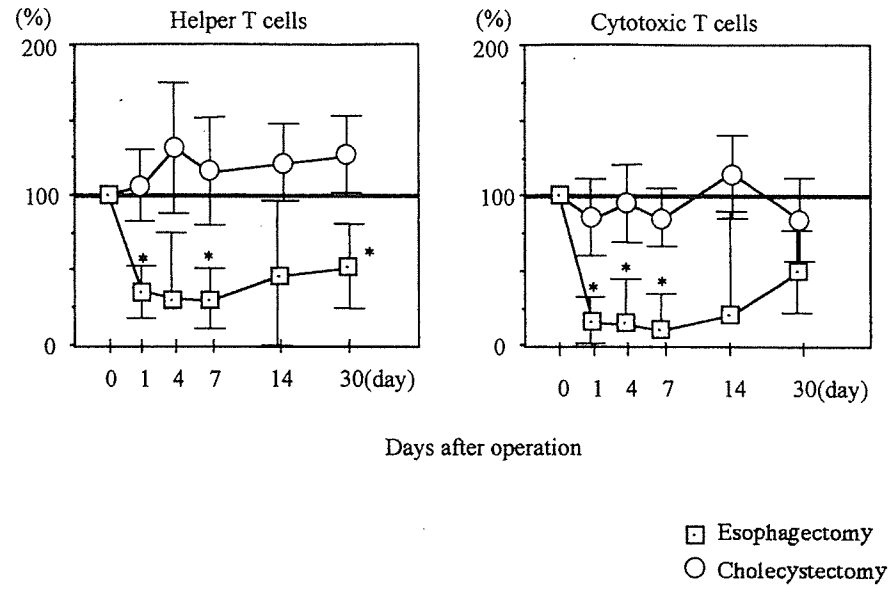

Figure 3. Postoperative changes in helper and cytotoxic T-cell subsets. PBMCs were postoperatively collected from patients who underwent transthoracic esophagectomy or open cholecystectomy, and helper and cytotoxic T-cell subsets were measured by flow cytometry. Significant differences, *p<0.05.

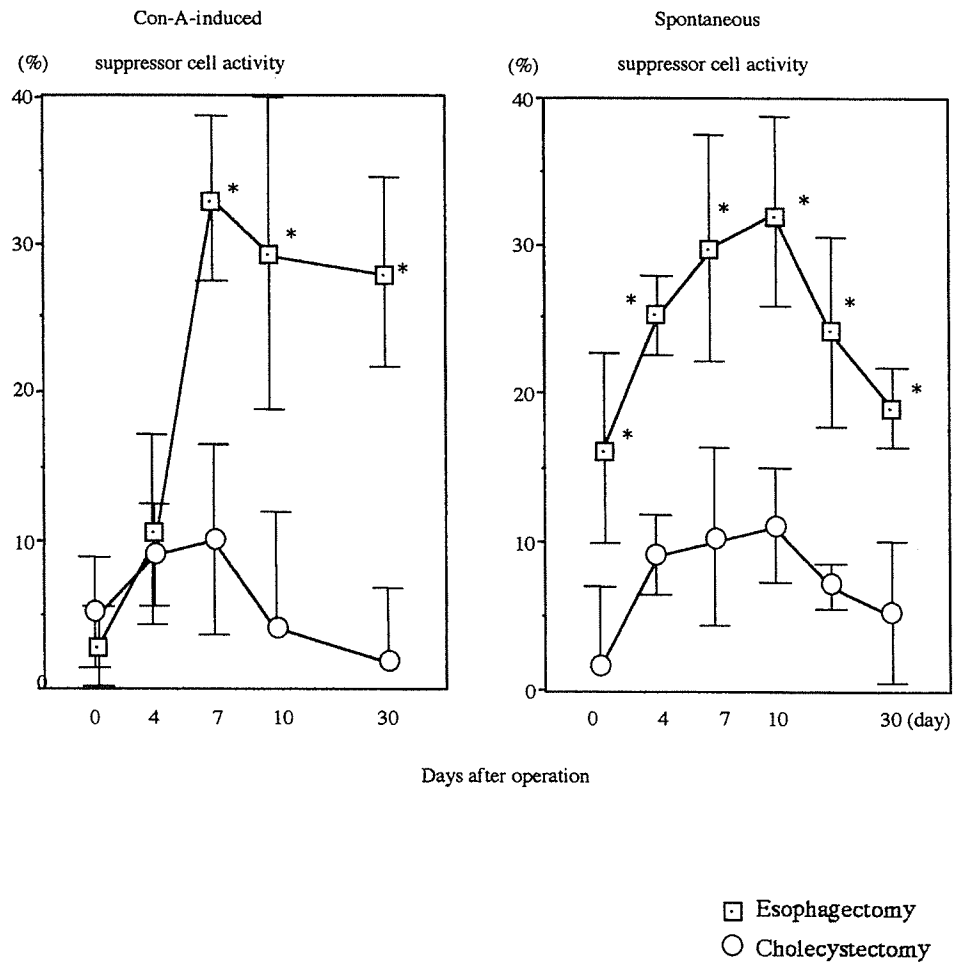

Figure 4. Postoperative changes in suppressor activities. PBMCs were postoperatively collected from patients who underwent transthoracic esophagectomy or open cholecystectomy, and suppressor activities were measured as described in Materials and methods. Significant differences, *p<0.05.

investigated (Fig. 4). Con-A-induced suppressor activity significantly increased in the EG group, peaked on day 7, then gradually decreased but still remained high on day 30 ($p<0.05$). Con-A-non-induced spontaneous suppressor

Table II. Esophageal cancer patients received postoperative adjuvant LAK therapy.

	LAK therapy	
	(-), n=15	(+), n=14
Gender		
Male	13	14
Female	2	0
Age, range (mean)	47-77 (61.8)	49-69 (56.8)
Stage		
0	4	1
1	0	1
2	1	2
3	6	6
4	4	4
LAK cell numder (range, median)	-	1.1-12.6 ($4.5x10^9$)
No. of transfer (range, median)	-	3-6 (4)

Table III. Postoperative infections after esophagectomy with or without LAK therapy.

	LAK	
Infection	(-), n=15	(+), n=14
(-)	10	12
(+)	5	2
Pulmonary	3	2
Wound	2	0

Patients who underwent transthoracic esophagectomy were treated either with or without postoperative LAK cell transfer. The incidence of postoperative pneumonia and wound infections was evaluated. The statistical difference between the LAK(+) and LAK(-) groups was p=0.09.

activity also increased significantly in the EG group, but peaked on day 10, and decreased thereafter ($p<0.05$). These changes were minimal in the CC group.

Patients with esophageal cancer who received postoperative adjuvant LAK therapy. Twenty-nine patients who underwent transthoracic esophagectomy were randomly treated postoperatively with or without LAK cell transfer. Patient characteristics are shown in Table II. There were no significant differences between LAK (+) and (-) groups in terms of gender, age or tumor stage. LAK cells were transferred safely postoperatively approximately four times with a total dose of $4.5x10^9$ cells (median).

Effects of postoperative LAK therapy on changes in helper and cytotoxic T-cell subsets. Effects of LAK cell transfer on the postoperative changes in helper and cytotoxic T cell subsets were studied (Fig. 5). Levels of the helper subset decreased postoperatively, as mentioned above for the LAK(-) group. This decrease in levels of the helper subset was almost abrogated with postoperative LAK cell transfer. As for the cytotoxic T-cell subset, postoperative LAK therapy restored the decrease in the subset, similarly to the effects of the helper subset. Significant differences were observed between LAK(-) and LAK(+) groups with regard to the percentage of postoperative helper T-cell and cytotoxic T-cell populations ($p<0.05$).

Postoperative infection after esophagectomy with or without LAK therapy. Postoperative pulmonary and wound infections were evaluated (Table III). In the LAK(-) group, 3 and 2 of 15 patients developed pneumonia and wound infection, respectively. In contrast, only 2 of 14 patients developed pneumonia in the LAK(+) group, and no wound infection was

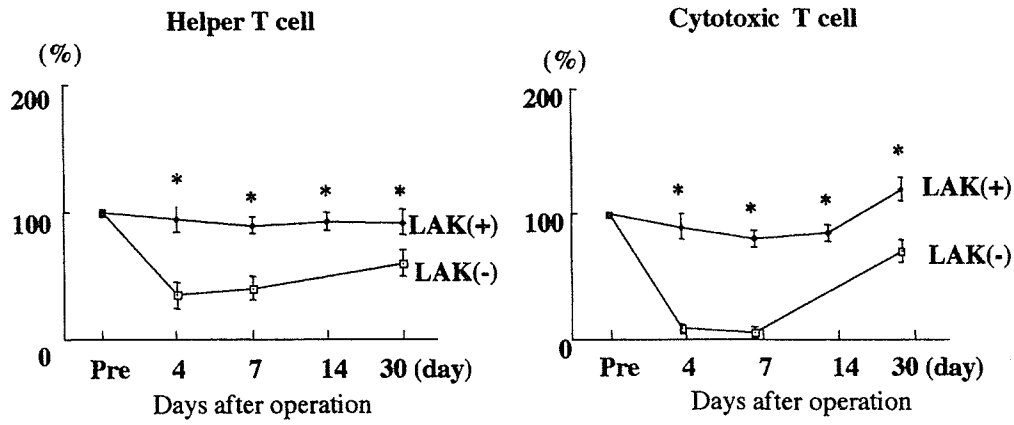

Figure 5. Effects of postoperative LAK therapy on changes in helper and cytotoxic T-cell subsets. LAK cells were prepared preoperatively. Patients who underwent transthoracic esophagectomy were treated either with or without postoperative LAK cell transfer. PBMCs were collected postoperatively, and helper and cytotoxic T cell subsets were measured in each group by flow cytometry. Significant differences, *$p<0.05$.

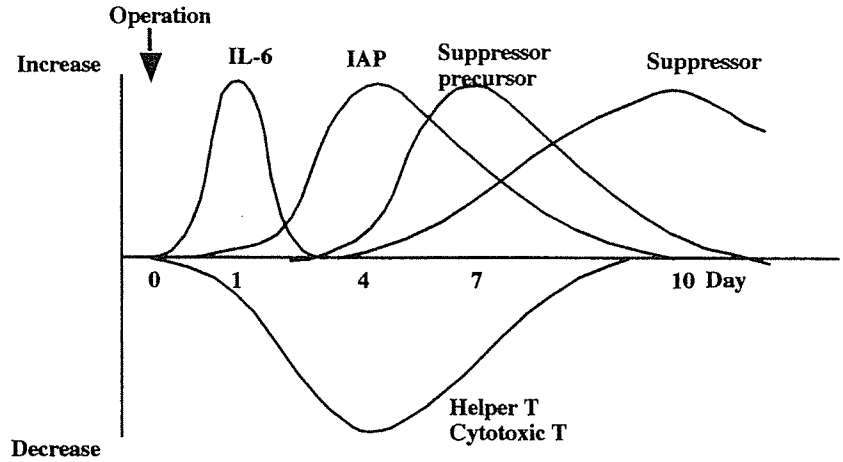

Figure 6. A proposal regarding the postoperative immunosuppression cascade. A postoperative immunosuppression cascade appears to exist that starts from increases in inflammatory cytokines, followed by increases in immunosuppressive proteins, decreases in helper and cytotoxic T-cell populations, and the development of suppressor cell activities, all of which contribute to the emergence of CARS in surgery for esophageal cancer.

observed. There was a trend of reductive effects on infections in the LAK(+) groups (p=0.09).

Discussion

We investigated postoperative changes in immunological parameters in order to elucidate the influences of surgical stress and host responses after transthoracic esophagectomy. It was found that levels of proinflammatory cytokine IL-6 and anti-inflammatory cytokine IL-10 increased postoperatively and peaked on day 1, followed by an increase in acute-phase reactant protein IAP, peaked on day 4, with a profound decrease in levels of helper and cytotoxic T-cell subsets, followed by increases in Con-A-induced and spontaneous suppressor activities. IL-6 has been reported to induce acute-phase reactant proteins like C-reactive protein and α1-acid glycoprotein (18). IAP has been reported to be a member of the α1-acid glycoprotein family (19). IAP also has been demonstrated to down-modulate CD4 molecules on the lymphocyte surface, leading to the establishment of cancer-associated immunodepression, and to induce suppressor-inducer cells (20). Con-A-induced suppressor cell activity has been reported to indicate the presence of suppressor precursor cells that mature in response to stimulation with suppressor inducer cells (17). Based on the above, we propose the existence of a postoperative immunosuppression cascade consisting of increases of cytokines, immunosuppressive proteins, decreases in helper and cytotoxic T-cell populations, and the development of suppressor T-cells (Fig. 6). All of these are triggered by the increase in inflammatory cytokines that induce humoral and cellular suppressive components.

In our postoperative immunosuppression cascade, suppressor cells are finally activated and differentiated. Recently, Sakaguchi *et al* (21) reported the existence of $CD4^+CD25^+$ new suppressor cells, designated as regulatory T (T-reg) cells. T-reg cells have been demonstrated to be involved in auto-immunity, tumor immunity, chronic infection and infertility. Murphy *et al* (22) have reported in a murine model that T-reg cells are involved in the suppression of innate immunity after the stress of injury. Although we did not fractionate lymphocytes by using CD4 antigen in the MLR experiments, the Con-A-induced or spontaneous suppressor activities described herein may include, to some extent, $CD4^+CD25^+$ T-reg cell activity, as these suppressor activities have been shown to reside in $CD4^+CD62L^+$ cells (23). Moreover, it has been reported that the development of T-reg cells requires transforming growth factor (TGF)-ß (24), and we have demonstrated TGF-ß production by $CD62L^+$ cells (23). Elias *et al* have reported the existence of an IL-6-TGF-ß regulation system in a human fibroblast model (25). These results suggest that T-reg cells may also be involved in the postoperative immunosuppression cascade in the human system. This hypothesis remains to be tested further.

The postoperative immunosuppression cascade described herein may be matched with the compensatory anti-inflammatory response syndrome (CARS). The CARS is a reciprocal situation triggered by SIRS after surgical stress (3,4). The CARS can cause remote infections, including pneumonia, based on profound immunodepression, where IL-10 plays an important role (5). IL-10 is a T-helper type 2 cytokine (11). Through insights into the pathophysiological mechanisms of SIRS and CARS, strategies for the use of T-helper type 1 cytokines of G-CSF (8), GM-CSF (9), IFN-γ (10) and IL-12 (11), have been studied with regard to the treatment of CARS, but the clinical benefits remain under investigation. In our study, CARS was thought of as a depression of functional lymphocytes, including helper T cells and cytotoxic T cells. This perspective may indicate that adoptive transfer of activated lymphocytes, for example LAK cells, is of use. It has been reported that IL-2-activated peripheral blood LAK cells consist of $CD4^+$ and CD8 cells as well as NK cells (26,27). Moreover, we have system for easily expanding LAK cells in our laboratory (13). Our preliminary clinical investigation showed that postoperative LAK cell transfer restores the decrease in helper and cytotoxic T-cell populations. Moreover, there has been a trend of reduction for post-operative remote infection and surgical site infection in the LAK therapy group. These results suggest that postoperative

adoptive transfer of LAK cells may be a novel clinical application for the treatment of CARS. A large clinical trial is now in progress for clarifying the anti-CARS effects of LAK therapy in patients with esophageal cancer.

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Locoregional immunotherapy of malignant ascites from gastric cancer using DTH-oriented doses of the streptococcal preparation OK-432: Treatment of Th1 dysfunction in the ascites microenvironment

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Abstract. Locoregional administration of the streptococcal preparation OK-432 is effective in treating malignant ascites from gastric cancer. In order to enhance the efficacy, we conducted a pilot study of locoregional immunotherapy for malignant ascites using host-oriented doses of OK-432. Moreover, action mechanisms of OK-432 were further explored in view of the T-helper type 1 (Th1)-Th2 concept. Gastric cancer patients with cytologically determined malignant ascites were locoregionally administered with OK-432. The dose of OK-432 was selected according to the delayed-type hypersensitivity (DTH) reaction levels to OK-432. Cytokine production profiles of ascites cells were determined using whole ascites assay by stimulation with OK-432. IL-10 mRNA expression was analyzed using RT-PCR. It was found that a positive clinical response was observed in 37 of the 51 (73%) patients with the DTH-oriented approach, showing a significantly higher efficacy than traditional dosage methods using empirical doses (31/58, 53%) ($p=0.0487$). The DTH-oriented administration of OK-432 produced adverse effects such as fever elevation ($p<0.0001$) and abdominal pain ($p=0.0013$) to a significantly lesser extent compared with the traditional treatment. Analysis of the action mechanism of OK-432 revealed that the DTH reaction in responders (19±6 mm) was stronger than that in non-responders (6±4 mm) ($p<0.0001$). Tumor necrosis factor (TNF)-α production of ascites cells was also higher in responders (3943±1247 pg/ml) than in non-responders (1217±939 pg/ml) ($p=0.0002$). There was a significant positive correlation ($p=0.0085$) between the levels of DTH reaction and TNF-α production of ascites cells, but not of blood cells. Responders appeared to polarize on the Th1 axis when clinical responses were plotted on Th1-Th2 dimensions according to the cytokine production profiles of TNF-α, IFN-γ, IL-4 and IL-6 of ascites cells. *In vitro* culture with IL-2 of ascites cells after OK-432 administration demonstrated an almost clonal expansion of $CD4^+$ lymphocytes, which produced TNF-α and IFN-γ, but did not produce IL-4 or IL-6. IL-10 mRNA expression was detectable in ascites cells from non-responders before treatment. These results suggest that the DTH-oriented locoregional administration of OK-432 may be both effective and less toxic in treating malignant ascites from gastric cancer, showing a possibility of the tailored immunotherapy for malignant ascites. Th1 dysfunction exists in the microenvironment of malignant ascites from gastric cancer, in which IL-10 may, in part, play a role. The up-regulation of Th1 responses by OK-432 may result in positive clinical responses. The DTH reaction to OK-432 may be a useful tool not only for predicting clinical response but also for selecting the optimal dose of OK-432.

Introduction

Malignant ascites often occurs as a principal clinical problem in primary or refractory cancer patients, and is associated with several objective and subjective symptoms such as anorexia, full sensation in the abdomen and dyspnea. If effective and practical treatments for malignant ascites were available for these terminally ill patients, it would offer them better quality of life and might possibly prolong the survival. Locoregional administration of various agents, including antineoplastic chemotherapeutic drugs (1,2), biological response modifiers for immunotherapy (2), and gene therapy agents (3) by paracentesis may be good clinical candidates for the treatment of malignant ascites due to its pharmacokinetic advances in drug delivery.

In the present study, we examine locoregional immunotherapy for malignant ascites using OK-432. OK-432 is a penicillin- and heat-inactivated lyophilized powder of *Streptococcus pyogenes* A3. In Japan, OK-432 has been approved for use in treating lymphangioma (4), in post-operative

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adjuvant therapy for several cancers in combination with anti-cancer chemotherapy (5,6), and in treating malignant effusions resulting from gastrointestinal and lung cancers (7). The standard approved dose of OK-432 for malignancies is 5 KE per body.

OK-432 has a capability to induce potent host-mediated immune responses and belongs to the category of biological response modifiers. The mechanism responsible for the anti-tumor activity of OK-432 has been studied both *in vitro* and *in vivo*. Uchida and Micksche (8) reported that intrapleural administration of OK-432 augmented ascites' natural killer (NK) cell activity and reduced ascites' NK suppressor cell activity. Katano and Torisu (9) demonstrated the induction of tumoricidal neutrophils by OK-432. In a previous study, we reported that the locoregional administration of OK-432 induced serial cellular infiltration of immunocompetent cells, including neutrophils, macrophages and lymphocytes, into the local cavity (7). Fujimoto *et al* (10) recently found that OK-432 stimulated IL-12 production to potentiate T-helper type 1 (Th1) responses. The terms Th1 and Th2 originally described in mouse model studies (11), and later were extended to studies on human immune systems (12,13). T-helper cells can be divided into two subpopulations, Th1 and Th2, according to the cytokine production profiles. Th1 produces interleukin (IL)-2, tumor necrosis factor (TNF) and interferon (IFN)-γ, and is involved in cellular immune responses including delayed-type hypersensitivity (DTH) reactions. On the other hand, Th2 produces IL-4, -5 and -6, and participates in humoral immune responses through IgE synthesis. Studies on the function of Th1 and Th2 have led to a greater understanding of many disorders, including autoimmune and neoplastic diseases (12,13).

We here conducted a pilot study of the locoregional immunotherapy for malignant ascites from gastric cancer using a host-oriented dose of OK-432. Further, we analyzed the clinical response in terms of Th1 and Th2 in order to address the mechanism of OK-432 immunotherapy. We will show that the up-regulation of Th1 responses by locoregional administration of OK-432 can result in a positive clinical response, and that the clinical response of patients with malignant ascites can be predicted with a DTH skin reaction, which may permit a host-oriented approach of determining an optimal dose of OK-432.

Materials and methods

Patients and locoregional immunotherapy using OK-432. Fifty-one gastric cancer patients with cytologically proven malignant ascites were enrolled in the study. The subjects were less than 80 years of age and had measurable ascites on sonographic examination and computed tomographic (CT) scans, an Eastern Cooperative Oncology Group performance status of 0-3, and a life expectancy of at least 3 months. Written informed consent was obtained from each patient before treatment. Ascitic fluid was removed by paracentesis before treatment, and a host-oriented dose of OK-432, described in detail below, was administered thereafter. OK-432 administration was once repeated on day 8 when no decrease of ascites was observed. Fifty-eight gastric cancer patients with malignant ascites who had been treated empirically with 5 KE of OK-432 served as a historical control. All of our subjects had been administered combination chemotherapy with 5-fluorouracil (350 mg/body, daily, continuously) and cis-platinum (5 mg/body, daily, intravenously), which had failed to reduce the ascites. Clinical responses were assessed on day 15 by cytological and sonographic examination together with CT scans. We then assigned our patients to one of two groups: responders, who showed disappearance or decrease of ascites with negative cytology for more than 1 month after the treatment; and non-responders, who had stable or increasing ascites even after the treatment. Toxicities associated with OK-432 immunotherapy were assessed according to the National Cancer Institute Common Toxicity Criteria Version 2.0, with the exception of fever elevation which was classified by degree: ≤37°C, 37-38°C, 38-39°C, and >39°C. Changes of performance status and subjective symptoms of full sensation in the abdomen and oral food intake were also evaluated before and after the treatment.

DTH skin reaction to OK-432 and its clinical dose. The DTH skin reaction test using OK-432 was performed in all patients prior to the treatment and doses of OK-432 were decided. In brief, 0.004 KE OK-432 dissolved in 0.02 ml saline was intradermally injected in the forearm and the diameter of redness was measured 24 h later. The dose of OK-432 for treatment was determined as follows: 10 KE for patients showing redness of 0-5 mm, 5 KE for those with 6-15 mm, 2 KE for those with 16-25 mm, and 1 KE for those with >26 mm of skin redness. One KE of OK-432 contains 0.1 mg of lyophilized streptococci.

Cytokine assay. Cytokine production potential was determined using whole blood/ascites assay in initial 20 patients consisting of 14 responders and 6 non-responders. Briefly, heparinized venous blood and ascites were collected prior to treatment and 0.5 ml of each sample was added to 5 ml of RPMI-1640 medium containing 0.1 KE/ml OK-432 (Chugai Pharmaceutical Company, Tokyo). Incubation was performed at 37°C in a 5% CO_2 incubator and supernatants were collected at 24 h, because preliminary experiments showed that cytokine production of blood cells and ascites cells peaked at 24 h when stimulated with 0.1 KE/ml of OK-432. IFN-γ and TNF-α were measured as Th1 cytokines, and IL-4 and -6 as Th2 cytokines by ELISA (R&D Systems, Minneapolis, MN).

Preparation of locoregional lymphocytes. Heparinized ascites cells of responder patients were obtained by paracentesis before and after treatment and pelleted. Cells were resuspended in RPMI-1640 medium and layered on 75/100% Ficoll-Conray gradient. After centrifugation at 400 g for 30 min, autologous tumor cells were collected from 75% interface and mononuclear cells from 100% interface. Mononuclear cells were washed 3 times and incubated in RPMI-1640 medium supplemented with 2% heat-inactivated autologous serum and 80 U/ml IL-2. Incubation was maintained with changing half the medium twice a week. On day 21, cells were harvested and subjected to flow cytometry, and culture supernatants were collected for measuring cytokine production by ELISA.

Table I. Patient characteristics.

	Empirical (n=58)	DTH-oriented (n=51)
Age	62±8	64±9
Gender (male/female)	41/17	32/19
P.S. (0/1/2/3)	4/21/28/5	4/19/24/4
Prior operation (yes/no)	52/6	46/5
Histology		
Differentiated	13	11
Undifferentiated	45	40
Concurrent metastases		
Liver	17	18
Lymph node	47	45

Flow cytometry. Fifty µl of the lymphocyte suspension ($5x10^5$) was incubated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE)-labeled monoclonal antibodies at 4°C for 45 min. Cells were washed twice and resuspended in RPMI-1640 medium. The monoclonal antibodies used were anti-Leu4a (CD3), -Leu3a (CD4), -Leu2a (CD8) and -Leu19 (CD56, Becton Dickinson, Mountain View, CA). Flow cytometric analysis was performed on Cytron (Ortho Diagnostic Systems, Inc., Raritan, NJ). After being adequately gated on lymphocytes using forward and side scatter, data collection was set up to stop when 10000 events had been analyzed.

IL-10 mRNA expression. Reverse transcription-polymerase chain reaction (RT-PCR) was performed to analyze IL-10 expression. In brief, total cellular RNA was extracted by acid guanidinium thiocyanate-phenol-chloroform extraction, and RNA samples were reverse-transcribed into cDNA with a random hexamer (14). PCR amplification of the cDNA was performed in a reaction mixture consisting of cDNA samples, Taq polymerase (Gibco-BRL, Grand Island, NY, USA) and the following primers: IL-10, 5'-AAGCTGAGAACCAAGA CCCAGACATCAAGGC-3' and 3'-AGCTATCCCAGAGCC CCAGATCCGATTTTGG-5' (15), and ß-actin (Stratagene, La Jolla, CA, USA). The reaction was carried out in a Perkin-Elmer Cetus thermal cycler (Perkin-Elmer Corporation, Eden Prairie, MN, USA) under conditions of 3 min denaturation at 94°C followed by 35 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C. After amplification, 8 µl of the reaction mixture was removed and analyzed by electrophoresis through 2.0% agarose gels in Tris-borate-EDTA buffer, and the gels were then stained with ethidium bromide. The expected lengths of the amplified cDNAs were 328 and 514 bp for IL-10 and ß-actin, respectively.

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

Table II. Comparison of clinical efficacies between empirical and DTH-oriented OK-432 immunotherapies for malignant ascites in gastric cancer patients.

Response	Empirical (n=58)	DTH-oriented (n=51)
Yes	31 (53)	37 (73)
No	27 (47)	14 (27)

Locoregional immunotherapy of malignant ascites was performed in gastric cancer patients using DTH-oriented dose of OK-432, and its clinical responses were compared with those of empirical control treatments. A statistical value was significantly different, p=0.0487. Parentheses indicate percentages.

Results

Patient characteristics. The characteristics of the 51 patients in the present study are listed in Table I. The patients had a mean age of 64±9 years, and included 32 males and 19 females, and showed performance status (P.S.) values of 0-3. Forty-six patients had undergone prior resection of the primary tumor. Eighteen and 45 patients had concurrent liver and lymph node metastases, respectively, as well as ascites detectable by CT scan. Gastric cancer patients (n=58) with malignant ascites, who had been previously treated with an empirical dose of OK-432 (5 KE), are also shown in Table I as a historical control group. No significant differences were observed in the patients' characteristics between the DTH-oriented group and the control group.

Clinical efficacy of locoregional immunotherapy for malignant ascites using OK-432. Clinical efficacy was evaluated by sonographic examination and CT scan (Table II). Positive responses were observed in 37 of 51 (73%) patients in the DTH-oriented group and in 31 of 58 (53%) patients in the control group in treating malignant ascites by locoregional immunotherapy using OK-432. There was a significant difference in response rate between the control and the DTH-oriented groups (p=0.0487).

Toxicity of locoregional immunotherapy for malignant ascites using OK-432. The DTH-oriented administration of OK-432 was assessed in terms of toxicity (Table III). Hematological toxicity was not observed in any patients analyzed except as an increase in white blood cell counts at approximately 12000-18000/mm^3, which decreased spontaneously within one week (data not shown). The most common constitutional symptom was fever elevation with temperatures of >37°C observed in the control group in 90% of patients treated by OK-432, and most notably in 14% of the control group, who suffered a fever of >39°C. In the DTH-oriented group, 71% patients showed a fever elevation of >37°C and <38°C, and no patients suffered a temperature of >39°C. There was a significant difference of fever elevation levels between the control and study groups (p<0.0001). Abdominal pain, the second most frequently observed adverse effect, was also

Table III. Comparison of adverse effects between empirical and DHT-oriented OK-432 immunotherapies for malignant ascites in gastric cancer patients.

Adverse effects	Empirical (n=58)	DHT-oriented (n=51)
Fever elevation (°C)		
<37	6 (10)	5 (9)
≥37, <38	17 (29)	36 (71)
≥38, <39	27 (47)	10 (20)
≥39	8 (14)	0
Abdominal pain		
Grade 1, 2	8 (14)	14 (27)
Grade 3	12 (21)	0

Locoregional immunotherapy of malignant ascites was performed in gastric cancer patients using DTH-oriented dose of OK-432, and its adverse effects were compared with those of empirical control treatments. Statistical values were $p<0.0001$ and $p=0.0013$ for adverse effects of fever elevation and abdominal pain, respectively. Parentheses indicate percentages.

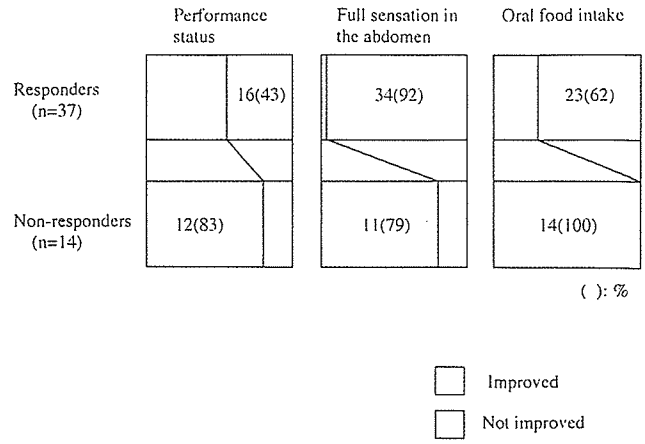

Figure 1. Clinical significance of locoregional immunotherapy for gastric cancer patients with malignant ascites. Gastric cancer patients with malignant ascites were treated with locoregional immunotherapy using OK-432, and improvement in the findings indicated, both subjective and objective, were compared between responders and non-responders.

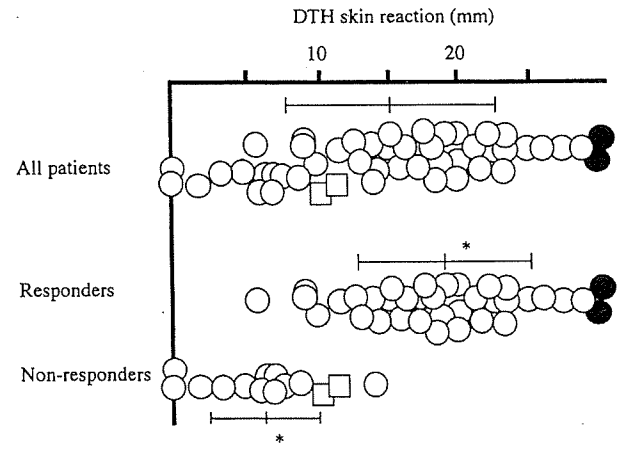

Figure 2. DTH skin reaction levels in gastric cancer patients with malignant ascites. A DTH skin reaction test using OK-432 was performed in gastric cancer patients with malignant ascites before OK-432 immunotherapy, and the diameter of skin redness was measured at 24 h and plotted. Patients who had skin redness with blister (•) and those with faint redness (□) are also shown. Significant difference from the value of responders, *$p<0.0001$.

examined. In the control group, 21% of patients showed grade 3 dull pain that disturbed their daily life for a couple of days after the treatment. In the DTH-oriented group, 27% of patients complained of grade 1 or 2 abdominal pain, but none suffered pain of grade 3. There was a significant difference in abdominal pain between the two groups ($p=0.0013$). Other toxicities experienced were of no more than grade 2 in the DTH-oriented group (data not shown).

Improvement of symptoms in responders. The significance of OK-432 immunotherapy was assessed by comparing performance status, full sensation in the abdomen and oral food intake between responders and non-responders (Fig. 1). Improvement in these three symptoms was found to be 43%, 93% and 64% in responders, and 17%, 17% and 0% in non-responders, respectively. There were significant differences between the presented values of responders and non-responders in full sensation of the abdomen and oral food intake, but not in performance status ($p<0.0001$, $p<0.0001$, $p=0.0986$, respectively).

DTH reactions to OK-432 and clinical responses. In order to address the background of responder patients to OK-432 immunotherapy, the DTH skin reaction to OK-432 was analyzed in patients with malignant ascites (Fig. 2). The redness of the DTH reaction differed from patient to patient within the range of 0-30 mm in diameter, with a mean ± SD of 15±8 mm. These skin reactions were clearly divided into two populations in view of clinical responses to OK-432 treatment. The redness diameters of the DTH reaction in responders and non-responders were 19±6 and 6±4 mm, respectively, and the DTH reaction of responders was significantly stronger than that of non-responders ($p<0.0001$). Moreover, blister formation was observed in 2 responders, while it was rather difficult to determine the redness margin in 2 non-responders because of its faintness.

TNF-α production of blood cells and ascites cells by OK-432. The cytokine production of blood cells and ascites cells by *in vitro* stimulation with OK-432 was measured with the whole blood assay and the whole ascites assay in patients treated with OK-432 immunotherapy (Fig. 3). The blood cells of responder patients produced significantly higher TNF-α than those of non-responders (1418±907 pg/ml vs. 533±422 pg/ml) ($p=0.036$). The ascites cells produced more TNF-α than blood cells by OK-432 stimulation, and the difference in TNF-α production between responders and non-

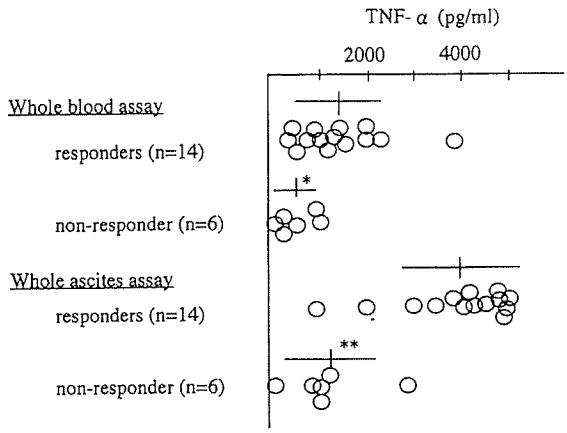

Figure 3. TNF-α production potential of blood and ascites cells stimulated with OK-432. Ascites fluids were collected from gastric cancer patients before OK-432 immunotherapy and diluted in medium, then stimulated with OK-432 for 24 h (whole ascites assay). TNF-α concentration in the supernatant was measured by ELISA. Values were significantly different between responders and non-responders, *p=0.036, **p=0.0002.

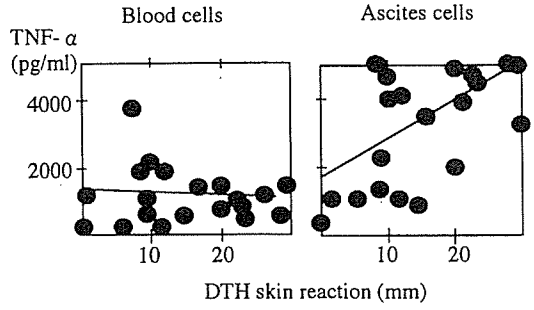

Figure 4. Relationship between DTH skin reaction levels and TNF-α production levels of blood cells or ascites cells. DTH skin reaction levels and TNF-α production levels of blood cells or ascites cells were measured in gastric cancer patients with malignant ascites before OK-432 treatment, and linear regression analysis was carried out to clarify relationship between both values. Regression coefficients were -0.036 (p=0.881) or 0.563 (p=0.0085) for relationships between DTH skin reaction levels and TNF-α production levels of blood cells or ascites cells, respectively.

responders was markedly augmented when evaluated by the whole ascites assay. Ascites cells from responder patients produced 3943±1247 pg/ml TNF-α, whereas those from non-responders produced 1217±939 pg/ml TNF-α. A significant difference was found between these two values (p=0.0002).

Relationship between levels of DTH response and TNF-α production. We next analyzed the correlation between levels of DTH reactions to OK-432 and TNF-α productions of blood cells or ascites cells stimulated with OK-432 *in vitro* (Fig. 4). We were unable to find any correlation between the DTH reaction and TNF-α production levels of blood cells stimulated with OK-432 (r=-0.036, p=0.881). We did, however, observe a significant positive correlation between the DTH reactions and TNF-α production levels of ascites cells by OK-432 stimulation, in which the correlation coefficient was calculated at 0.563 (p=0.0085).

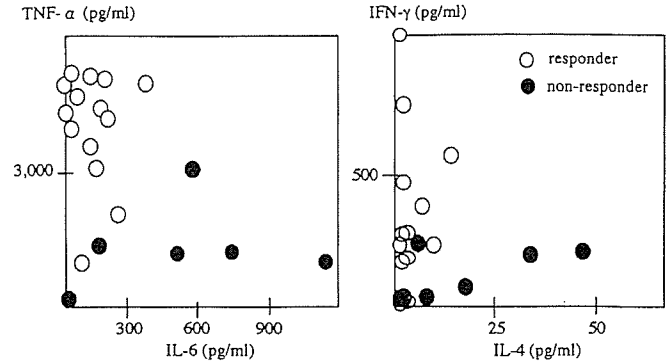

Figure 5. Comparison between clinical responses of OK-432 immunotherapy and Th1, Th2 cytokine production profiles of ascites cells by *in vitro* stimulation with OK-432. Cytokine production profiles, including TNF-α and IFN-γ as Th1 cytokines, and IL-6 and IL-4 as Th2 cytokines, of ascites cells from gastric cancer patients were determined by whole ascites assay using OK-432 stimulation before OK-432 immunotherapy. Clinical responses were plotted on a Th1-Th2 dimension as indicated.

Relationship between clinical responses and Th1/Th2 cytokines. Clinical responses were analyzed in detail in order to explore the mechanisms of OK-432 immunotherapy by using the Th1/Th2 concept based on cytokine production profiles of ascites cells of TNF-α, IFN-γ, IL-4 and IL-6, which were measured by means of the whole ascites assay using OK-432 (Fig. 5). When the clinical responses were plotted on a TNF-α/IL-6 dimension, responder patients appeared to polarize on the TNF-α axis, while the non-responder patients deviated on the IL-6 axis. A similar result was observed when the data were analyzed in the IFN-γ/IL-4 dimension, in which the responders polarized on the IFN-γ axis but the non-responders did not.

Preferential expansion of CD4⁺ Th1 lymphocytes. Ascites lymphocytes of 3 responders were stimulated *in vitro* with IL-2 and further characterized by the phenotype analysis and cytokine production analysis (Table IV). More than 89% of lymphocytes predominantly expressed $CD3^+$ and $CD4^+$ phenotypes, but CD8 and CD56 populations were around 15%. These $CD3^+CD4^+$ lymphocyte lines were found to produce predominantly TNF-α and IFN-γ but little or no IL-4 or IL-6.

IL-10 mRNA expression of ascitic cells. Finally, the expression of Th2 cytokine IL-10 was examined at the mRNA level (Fig. 6). Among the 13 patients examined, 10 of the 11 responders to OK-432 immunotherapy showed no expression of IL-10 mRNA in ascites cells before treatment (Fig. 6, lanes 1-10). However, both non-responders, as well as one responder, demonstrated positive bands of IL-10 mRNA expression in ascites cells prior to treatment (Fig. 6, lanes 11-13).

Discussion

We have demonstrated here the efficacy of locoregional immunotherapy for malignant ascites in gastric cancer patients using the streptococcal preparation OK-432. Approximately