Effects of Hochu-ekki-to on cytotoxicity and cell surface adhesion molecules of activated T lymphocytes

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Abstract

In the present study, we demonstrated that Hochu-ekki-to (補中益気湯 ; TJ-41) could enhance the cytotoxic activity of human activated T lymphocytes, which had been enriched by Ficoll-Paque density separation and incubated with anti-CD3 monoclonal antibodies (MoAb) and recombination human IL-2 (rhIL-2), against T98G glioblastoma and Molt-4 T lymphoblastoma cells *in vitro*. TJ-41 was found to possess these properties in a dose-dependent manner.

We also studied whether TJ-41 may affect the expression of CD11a/LFA-1, CD4, CD8, CD25 and HLA-DR cell surface molecules. The results showed that TJ-41 clearly and selectively affected only CD11a/LFA-1 expression and that this effect was not seen in CD4, CD8, CD25 or HLA-DR expression. When CD11a/LFA-1 expression was enhanced by TJ-41, cytotoxicity was also increased. Cytotoxicity of activated T lymphocytes against T98G glioblastoma cell was inhibited by anti-CD11a/LFA-1 monoclonal antibody. IFN-γ and IL-4 content in the supernatant of activated T lymphocytes was also increased by TJ-41. Similar results were observed in the activated T lymphocytes of cancer patients. In our study, Astragalus Root and Atractylodes Lancea Rhizome increased cytotoxicity to a higher degree than the other raw herbs cotained in TJ-41.

Key words Activated T lymphocytes, Hochu-ekki-to (Bu-Zhong-Yi-Qi-Tang, 補中益気湯), Cytotoxicity, T98G glioblastoma cell line, Molt-4 T lymphoblastoma cell line, CD11a/LFA-1, CD4, CD8, CD25, HLA-DR.

Abbreviations NK, Natural Killer ; PBS, Phosphate buffered saline.

Introduction

Hochu-ekki-to (補中益気湯 ; TJ-41) is a formula of Kampo herbal medicines applied in Japan as an effective biological response modifier for augmenting host immune function. It has already been shown to possess anti-tumor activity and to reduce the side-effects of anti-cancer agents.[1,2] In animal experiments, the oral administration of TJ-41 with gastric gavage significantly enhanced specific anti-tumor activity against Meth A at rechallenge on day 9. The tumor-draining lymph node cells of the mice treated with TJ-41 showed anti-tumor activity against Meth A. In a cytolytic assay, the anti-Meth A specific cytolytic T lymphocyte activity was not detected in the spleen cells of mice bearing Meth A and treated with TJ-41. The oral administration of TJ-41 enhanced the natural killer (NK) activity of the spleen cells in healthy mice but could not improve the decreased NK activity of spleen cells from the tumor bearing mice. These results indicate that the oral administration of TJ-41 to tumor-bearing mice may thus be able to enhance concomitant anti-tumor immunity through the augmentation of cytostatic activity.[3] On the other hand, the splenic NK cells of TJ-41-treated WKA rats

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showed enhanced cytotoxicity against tumors involving NK-sensitive YAC-1 targets. TJ-41 did not increase the splenic NK cell population, but it appeared that TJ-41 could potentiate the cytotoxic capability of NK cells. These data indicate that TJ-41 may be useful for prophylactic immunotherapy of cancer. One of the mechanisms of the potent inhibitory effect of TJ-41 on tumor growth might be explained by its enhancement of NK activity.[4]

The adhesion molecules expressed on T lymphocytes were crucial for stabilizing cell-cell interactions; they were first thought to be accessory molecules that simply joined one cell to another. Recently it has been realized that these molecules also transmit signals from outside to inside the cell.[5] CD11a/LFA-1 is a major ligand for intercellular adhesion molecule-1 (ICAM-1). It is expressed on a variety of cells in inflammatory lesions and induces cytokines.

Anti-tumor activity was studied in many aspects of the investigation area, but the precise mechanism was still unclear. A new approach to the treatment of cancer, adoptive immunotherapy using cytolytic T lymphocytes, has recently engendered great interest as a potential therapy for malignancies. A feasible method for expansion of peripheral blood lymphocytes by culture with immoblized anti-CD3 monoclonal antibody and interleukin-2 has been used in adoptive immunotherapy of cancer patients in a clinic.[6] In this study, in order to clarify the immunopharmacological mechanisms of Kampo medicines affecting cytolytic T lymphocytes, we analyzed the effects of Kampo medicine on cytotoxicity as well as the relation between cytotoxicity and CD11a/LFA-1, CD4, CD8, CD25, HLA-DR expression of activated human T lymphocytes.

Materials and Methods

Reagent and antibodies : The formulae of Kampo herbal medicines and raw herbs were provided by Tsmura Co. (Japan) and Uchida Wakan-Yaku Co. Ltd. (Tokyo, Japan), respectively. Human rhIL-2 and anti-CD3 MoAb were provided by the National Cancer Center. Anti-human CD11a/LFA-1, anti-human CD4, anti-human CD8, anti-human CD25, anti-human HLA-DR were purchased from PharMingen (Torreyana Road, San Diego, CA, USA).

Preparation of activated T lymphocytes : Peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood of healthy donors and patients by Ficoll-Paque density separation. The cells were cultured for 3 days with 700 unit/ml of rhIL-2 in a flask coated with anti-CD3 MoAb. After fresh medium was exchanged, Kampo medicine was added at a certain concentration during incubation 48 hr. In dose-dependent assay, TJ-41 was added at final concentrations of 100, 50, 25 and 10 μg/ml and cultured for 2 days at 37°C in a 5 % CO_2 incubator. After washing twice in fresh medium, these effector cells were used for the cytotoxicity and Flow cytometric analysis.

Cell lines : T98G cells and Molt-4 cells were maintained at the Department of Bioregulatory Function of the Graduate School of Medicine, University of Tokyo.

Flow cytometric analysis : Flow cytometric analysis of lymphocyte surface phenotypes was carried out by direct immunofluoresecence using an EPICS XL (Coulter Cytometry Co., Hialeah, FI, USA). Lymphocytes (1×10^6cells) were incubated for 1hr at 4°C with 10 μl of appropriate dilution of fluorescein-isothiocyanate-(FITC)-conjugated or phycoerythrin (PE)-conjugated monoclonal antibodies against CD11a/LFA-1, CD4, CD8, CD25 and HLA-DR. A fluorescence histogram of at least 5000 counts was collected for each sample.

Cytotoxic assay : Cytotoxicity against T98G glioblastoma was determined by colormetric assay,[7] which was slightly modified in our laboratory. Briefly, cell (100 μl) were added to wells of 96-well flat bottom microtitre plates (Falcon Labwere, Oxnard, CA) and incubated for 1hr at 37°C. Activated T lymphocytes 100 μl were added as effector cells to the same plate. The effector cell to target cell ratio was fixed at 4 : 1. After incubation for 18 hrs, the plate was washed twice and 200 μl of fresh medium and 20 μl of alamar blue solution were added. After 4hrs, the optical density (OD) was measured by microplate reader (BioRad Laboratories, Alfred Nobel Drive, Hercules, CA, USA) at 570 nm. The mean value of OD of 5-6 wells was used for calculating the % cytotox-

icity. The equation is as follows

$$\left(1 - \frac{\text{Test OD value-blank OD value}}{\text{Target OD value-blank OD value}}\right) \times 100$$

Inhibition assay : Activated T lymphocytes cell were preincubated with anti-CD11a/LFA-1 monoclonal antibody (2 μg/ml) 1hr before Kampo medicine was added at a certain concentration during incubation 18 hr. After incubation 18 hr, the activated T lymphocytes cell was washed twice, cytotoxicity was measured as described above.

In cytotoxicity assay for Molt-4 cell as a target cell experiment, cytotoxicity was determined in triplicate at an effector-to-target ratio of 10 : 1 against Molt-4 lymphoblastoma in an enzyme-release assay.[8] 1×10^6 activated T lymphocytes in 0.1 ml were added to appropriate wells of a V-bottom 96-well plate (Costar Co., Cambridge, MA. USA) and this was followed by the addition of 0.1 ml of target Molt-4 lymphoblastoma cells at 1×10^5 cells/ml. The plate was centrifuged (250×g. for 5 minutes) and incubated for 4 hours at 37°C in 5 % CO_2 to measure released lactate dehydrogenase, A Cyto Tox 96 non-radoactive cytotoxicity assay kit (Promega Co., Madison, WI. USA) was used according to the instructions of the manufacturer.

Cytokine assay in the supernatants : Activated T Lymphocytes (1×10^6 cell/ml) which had been treated with TJ-41 were cultured in anti-CD3 MoAb-coated 24-well flat bottom microtitre plates at 37°C in a humidified 5 % carbon dioxide atmosphere for 48 hrs. The supernatant was collected after centrifugation. IL-12, IFN-γ and IL-4 were assayed by using enzyme-linked immunosorbent assay (ELISA) kits (Bio Source International Inc., Flynn Road, Camarillo, CA,USA).

Results

In vitro cytotoxicities of activated T Lymphocyte treated Kampo medicines against T98G cells

In the experiment, the Kampo formulae HET (Hochu-ekki-to ; 補中益気湯), JTT (Juzen-taiho-to ; 十全大補湯), NYT (Ninjin-yoei-to ; 人参養栄湯), SBT (Shimbu-to ; 真武湯), SST (Sho-saiko-to ; 小柴胡湯), HJG (Hachimi-jio-gan ; 八味地黄丸), NJT (Ninjin-to ; 人参湯) were tested in the screening of cytotoxicity assay. Figure 1 shows the *in vitro* cytotoxicites

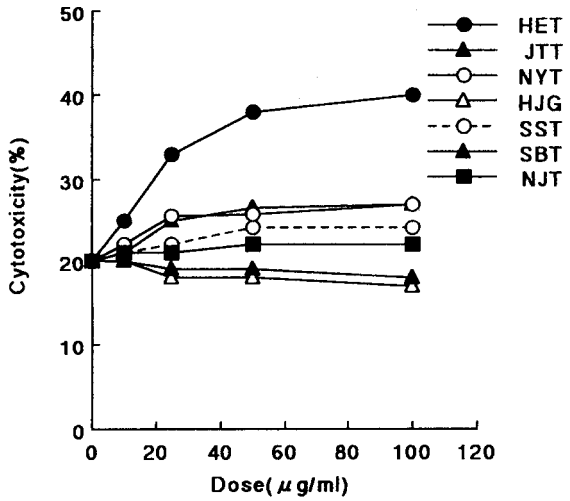

Fig. 1 Effect of Kampo formulae on cytotoxicity of activated T lymphocytes against T98G glioblastoma cell line (%). HET : Hochu-ekki-to, 補中益気湯, JTT : Juzen-taiho-to, 十全大補湯, NYT : Ninjin-yoei-to, 人参養栄湯, HJG : Hachimi-jio-gan, 八味地黄丸, SST : Sho-saiko-to, 小柴胡湯, SBT : Shimbu-to, 真武湯, NJT : Ninjin-to : 人参湯. Cytotoxicity was measured by Alamar bule assay at the ratio of 4 : 1 in triplicate well.

of the series of 7 formulae of Kampo herb medicines against T98G cells. Activated T lymphocytes treated with TJ-41 showed significantly higher cytotoxicity than cells treated with other Kampo medicines at a final concentration of 100 μg/ml.

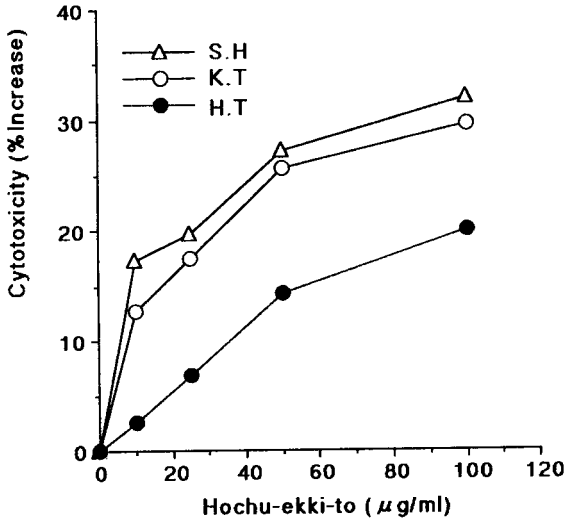

Fig. 2 Effect of Hochu-ekki-to on cytotoxicity of activated T lymphocytes from healthy donors against T98G glioblastoma cell (% increase).

Cytotoxicity of activated T lymphocytes treated with TJ-41

Because the cytotoxicity of activated T lymphocytes treated with TJ-41 was the most potent among the tested Kampo medicines, we examined the dose-dependent effects of TJ-41 on the cytotoxicity of activated T lymphocytes from 3 healthy donors. As shown in Figure 2, TJ-41 (range from 10-100 μg/ml) augmented the cytotoxicity of activated T lymphocytes from all healthy donors examined in a dose-dependent manner.

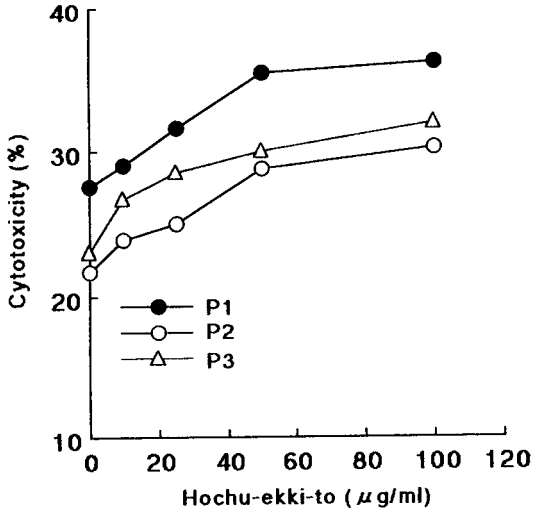

Fig. 3 Effect of Hochu-ekki-to on cytotoxicity of activated T lymphocytes from cancer paitents against T98G glioblastoma cells (%).

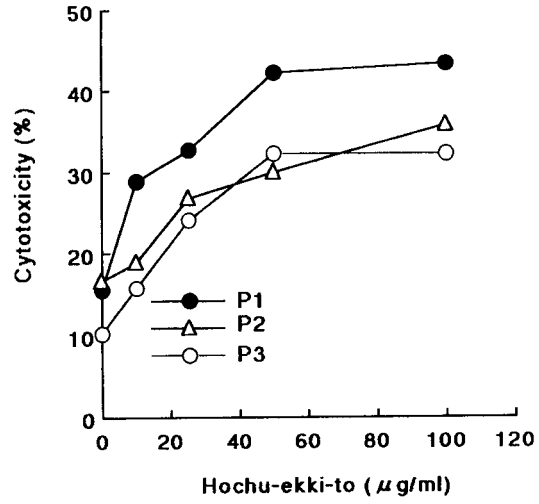

Fig. 4 Effect of Hochu-ekki-to on cytotoxicity assay of activated T lymphocytes from cancer patients against molt-4 lymphoblastoma cells (%).

Cytotoxicity assay was also performed with activated T lymphocytes from cancer patients. In this case, both adherent T98G glioblastoma cells and Molt-4 T lymphoblastoma cells in suspension were used as target cells. Figures 3 and 4 show that TJ-41 significantly augmented cytotoxicity against both T98G cells and Molt-4 cells.

Surface phenotypes of Lymphocytes treated with TJ-41

For studying the mechanism of the enhancement of cytotoxicity by TJ-41, the phenotypes (CD4, CD8, CD25, HLA-DR and CD11a/LFA-1) of the activated T lymphocytes treated with TJ-41 were determined by flow cytometry. The results show that TJ-41 insignificantly affected the phenotypes of CD4, CD8, CD25 and HLA-DR of activated T lymphocytes (Table I).

The results for the phenotypes of CD11a/LFA-1 of activated T Lymphocytes from healthy donors and cancer patients are shown in Figure 5 and Figure 6,

Table I Phenotypic analysis of activated T lymphocytes treated Hochu-ekki-to

	Dose (μg/ml)	Positive cells (%)			
		CD4	CD8	HLA-DR	CD25
S.H.	0	25.2	59.6	40.1	77.0
	10	24.6	60.9	42.1	77.0
	25	n.t	60.9	42.1	78.1
	50	25.2	57.4	43.2	79.6
	100	24.6	56.9	40.1	80.4
K.T.	0	23.9	67.8	42.4	76.1
	10	23.0	68.2	42.2	76.3
	25	21.2	68.1	42.1	77.1
	50	21.1	64.8	43.1	76.3
	100	23.9	67.8	42.4	77.1
H.T.	0	28.0	62.3	42.1	70.2
	10	30.6	n.t	42.1	70.1
	25	30.6	62.1	41.5	69.7
	50	30.1	60.7	42.1	71.1
	100	31.1	62.5	43.2	71.3

n.t=not tested

The phenotypes of the activated T lymphocytes treated Hochu-ekki-to were examined by flow cytometry in 3 healthy donors.

respectively. The results show that the phenotypes of CD11a/LFA-1 of both healthy donors and cancer patients were significantly increased by TJ-41(dose range 10-100 μg/ml.).

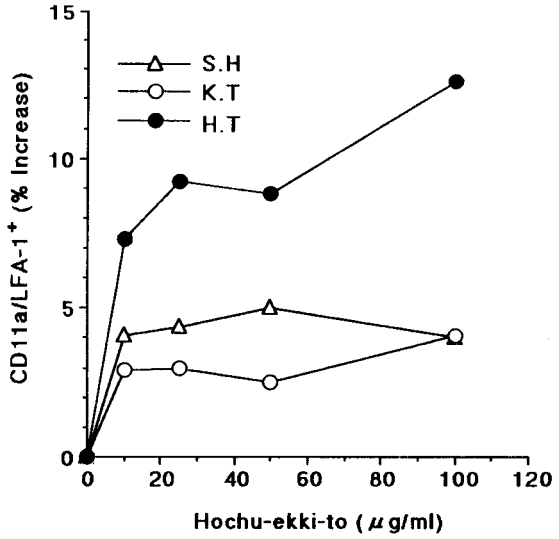

Fig. 5 Effect of Hochu-ekki-to on the expression of CD11a/LFA-1 on activated T lymphocytes from healthy donors (% increase).

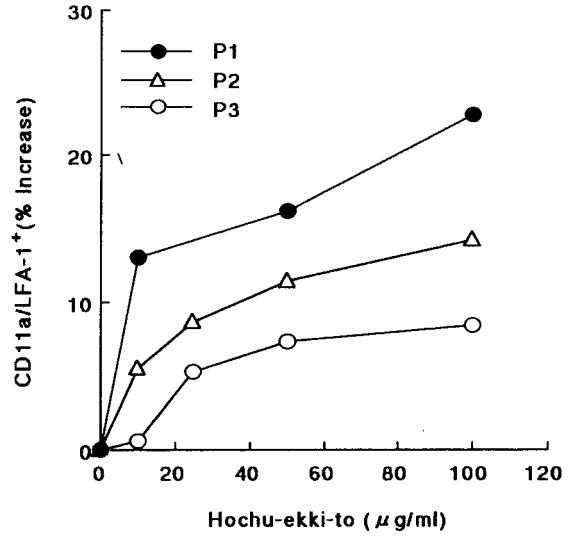

Fig. 6 Effect of Hochu-ekki-to on the expressiom of CD11a/LFA-1 on activated T lymphocytes from cancer patients (% increase)

Table II Cytokine production by activated T lymphocytes treated Hochu-ekki-to

	Dose (μg/ml)	INF-γ	IL-4	IL-12
S.H.	100	205.6	0.56	0.13
	0	186.1	0.20	0.14
K.T.	100	206.1	0.60	0.40
	0	199.1	0.10	0.15
H.T.	100	204.3	0.44	0.36
	0	185.9	0.27	0.13

Cytokine production by activated lymphocytes was measured. Activated T lymphocytes was induced from the three healthy donors. Cytokine production of activated T lymphocytes including INF-γ, IL-4 and IL-12.

Cytokine production

The cytokine production by activated T lymphocytes from healthy donors treated with TJ-41 was studied. As shown in Table II, IFN-γ and IL-4 production of activated T lymphocytes was augmented by TJ-41. IL-12 was insignificantly increased.

The inhibition of cytotoxicity of activated T lymphocytes treated with TJ-41 was cultured with anti-CD11a/LFA-1 antibody

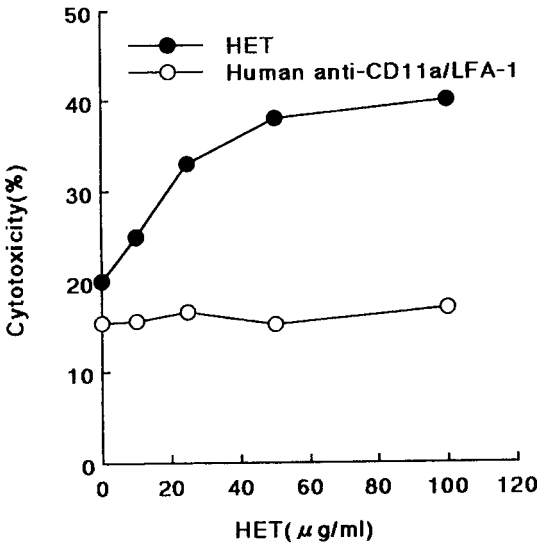

Fig. 7 Inhibition of the cytotoxicity of activated T lymphocytes for T98G glioblastoma cell line. Activated T lymphocytes were preincubated with anti-CD11a/LFA-1 monoclonal antibody, and the percent inhibition of cytotoxicity was calculated. All experiments were performed in triplicate.

To evaluate the role of CD11a/LFA-1 in cytotoxicity, we performed an inhibition assay. Cytotoxicity of activated T lymphocytes against T98G glioblastoma cell was inhibited by anti-CD11a/LFA-1 monoclonal antibody. The result is shown in Figure 7. It was demonstrated that CD11a/LFA-1 may contribute to the regulation of cytotoxicity of activated T lymphocytes treated with TJ-41.

Cytotoxicity of raw herbs of Hochu-ekki-to

Hochu-ekki-to consists of Astragalus Root (黄耆), Atractylodes Lancea Rhizome (蒼朮), Ginseng Root (人参), Japanese Angelica Root (当帰), Bupleurum Root (柴胡), Jujube Fruit (大棗), Citrus Unshiu Peel (陳皮), Glycyrrhiza Root (甘草), Cimicifuga Rhizome (升麻) and Ginger Rhizome (生姜). Figure 8 shows dose-dependent cytotoxicity of the series of 10 raw herbs against Molt-4 cells. Astragalus Root and Atractylodes Lancea Rhizome showed significantly higher cytotoxicity than the other herbs contained in TJ-41 at a final concentration of 100 μg/ml.

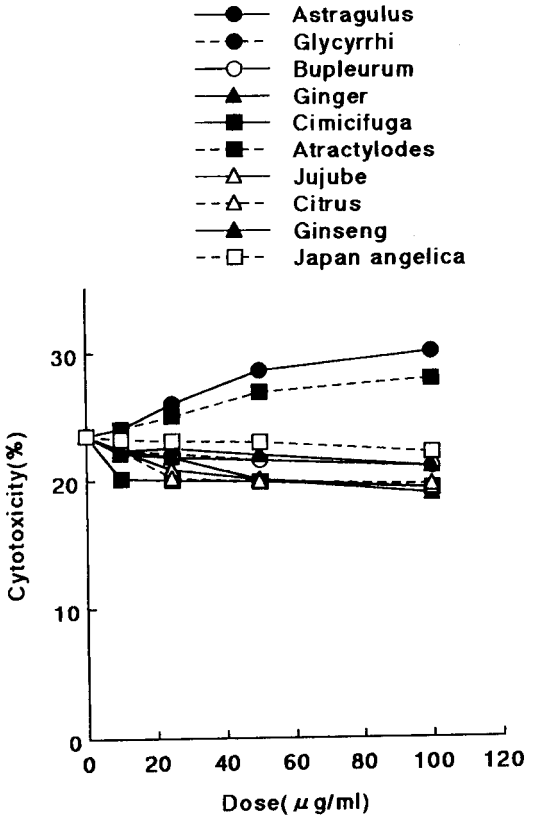

Fig. 8 Effect of the extracts of each herb contained in Hochu-ekki-to on the cytotoxicity of activated T lymphocytes against Molt-4 cells.

Discussion

In the present study, it was shown that TJ-41 enhanced the cytotoxicity of activated T lymphocytes taken from both healthy subjects and cancer patients. TJ-41 also enhanced the cytotoxicity against both T98G glioblastoma and Molt-4 lymphoblastoma cells. Flow cytometric analysis showed that TJ-41 did not significantly influence phenotypes of CD4, CD8, CD25 or HLA-DR of activated T lymphocytes, but significantly enhanced the expression of cell surface adhesion molecules CD11a/LFA-1. This enhanced activity was found in a dose-dependent manner. IFN-γ and IL-4 content of activated T lymphocytes treated with TJ-41 was augmented, but IL-12 content was not significantly changed.

The activated T lymphocytes derived from different donors differed in the increment of CD11a/LFA-1 expression induced by TJ-41. We found that the lower the inital levels of CD11a/LFA-1 lymphocytes, the more the level was increased by TJ-41.

Some biological response modifiers were recently tested for the ability to induce in vitro autologous tumor-killing activity in human cancer patients. Peripheral blood lymphocytes and tumor-infiltrating lymphocytes demonstrated various levels of cytotoxicity against autologous, freshly isolated tumor cells. When peripheral blood lymphocytes and tumor-infiltrating lymphocytes were cultured overnight with biological response modifiers, autologous tumor killing activity was induced in previously non-reactive and augmented in previously reactive cases.[9-11] In the present study, the cytotoxicity assay revealed that activated T lymphocytes exhibited a high level of killing activity against both T98G glioblastoma and Molt-4 lymphoblastoma cells, and that this the cytotoxicity was augmented by TJ-41. The results showed clearly that TJ-41, which is known to have a strong biological response modifying function and is expected to produce clinical improvement and prolongation of survival in treated cancer patients, has considerable clinical effectiveness.

It has recently become apparent that several of

the molecules involved in leukocyte adhesion also serve as signalling molecules. LFA-1, ICAM-1 and LFA-3 provide signals that regulate the lytic ability of cytotoxic T lymphocyte effectors.[12] Our experiment showed that activated T lymphocytes treated with TJ-41 expressed considerably augmented CD11a/LFA-1. Cytotoxicity of activated T lymphocytes against T98G glioblastoma cell was inhibited by anti-CD11a/LFA-1 monoclonal antibody. These data suggest that TJ-41 augmented cytotoxicity by enhancing the expression of CD11a/LFA-1 and regulating the signalling and adjustment between effector and target cells. Though TJ-41, at any concentration tested, did not affect the expression of CD4, CD8, CD25 and HLA-DR on the lymphocytes after activation by anti-CD3/rhIL-2, it selectively enhanced the expression of CD11a/LFA-1. Signal transmission enhanced by up-regulation of CD11a/LFA-1 may contribute to the regulation of cytotoxicity of activated T lymphocytes. In this study, Astragalus Root and Atractylodes Lancea Rhizome augmented cytotoxicity more than the other raw herbs in TJ-41. A further appoach to clarify the effect of TJ-41 would be to find active components which may be contained in Astragalus Root or Atractylodes Lancea Rhizome and to investigate the mechanism of cytotoxicity enhanced by surface molecules.

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和文抄録

補中益気湯などの補剤には担癌宿主の全身状態を改善し，細胞性免疫反応を増強する効果が認められると近年報告されている。補剤の宿主免疫反応への作用機序を明らかにする目的でヒト活性化T細胞を用いて細胞障害活性及び各種表面マーカーに対する補中益気湯の作用を検討した。

補中益気湯，十全大補湯，人参養栄湯，八味地黄丸，小柴胡湯，真武湯および人参湯の活性化T細胞に対する影響を検討した結果，補中益気湯原末のみに10-100 μg/mlの濃度で標的細胞障害活性を用量に高める効果が認められた。細胞表面抗原の検討では，補中益気湯はCl1a/LFA-1陽性細胞を増加させた。Cl1a/LFA-1は細胞障害活性を高めるよりも低濃度補中益気湯で誘導された。補中益気湯を作用させる前のCl1a/LFA-1発現が低いほど補中益気湯によるCl1a/LFA-1の増加率が高い傾向が認められた。さらに，補中益気湯の構成生薬について検討した結果，黄耆および蒼朮が標的細胞障害活性を高める効果が認められた。

Cl1a/LFA-1は白血球細胞表面接着分子lymphocyte function associated antigen-1 (LFA-1) のマーカーでありリンパ球とそのリガンドであるICAM-1との接着を促進する。LFA-1は活性化T細胞が標的細胞を認識し，細胞障害反応を起こす際に必須の分子として注目されている。補中益気湯による宿主免疫反応増強作用の一部はLFA-1誘導作用が関与しているものと考えられる。

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