Thesis

Effects of Traditional Chinese Medicine, Ekki-Youketsu-Fusei-Zai, on Survival and NK Cell Function of Tumor-Bearing Mice and on Cytokine Production by Murine Macrophage Cell Line J774.1

Department of Microbiology, Saitama Medical School
Moroyama, Iruma-gun, Saitama 350-0495, Japan

(Supervisor: Prof. Dr. Toshitaka Akatsuka)

Hong Deng

Abstract: We examined anti-tumor effects of the Traditional Chinese Medicine (TCM), Ekki-Youketsu-Fusei-Zai (EYFZ), on survival, tumor size, body weight, and natural killer (NK) cell activity of tumor-bearing mice by using a tumor cell line, colon-26. A significant life-prolonging effect was found, when EYFZ was orally administrated for 28 days, which started just after the subcutaneous implantation of colon-26 cell line. In addition, an oral administration of EYFZ inhibited the tumor growth and the loss of body weight in tumor-bearing mice. The significant increases in splenic NK cell activity of the tumor-bearing mice were also induced by oral administration of EYFZ. To elucidate the mechanisms of these anti-tumor effects, we further investigated the effect of EYFZ on cytokine production by murine macrophage-like cell line, J774.1 cells originating from BALB/c mice. In this experiment, we examined 6 kinds of cytokine mRNA expression by J774.1 cell in response to EYFZ-stimulation. RT-PCR revealed that both interleukin-12 (IL-12)p35 and IL-12p40 mRNA expression were induced by EYFZ treatment. Moreover, EYFZ promoted the secretion of IL-12 from J774.1 cells. These results strongly suggest that EYFZ has the anti-tumor effects on colon-26 implanted mice via augmentation of NK cell activity *in vivo* and induce the functional activation of J774.1 cells *in vitro*.

Keywords: Traditional Chinese Medicine (TCM), Ekki-Youketsu-Fusei-Zai (EYFZ), anti-tumor activity, natural killer (NK) cell activity, murine macrophage cell line J774.1, IL-12 mRNA expression, IL-12 production.

Introduction

Some kinds of crude drugs of Traditional Chinese Medicine (TCM) have been so far useful for cancer patients as both an anti-tumor drug and an adjuvant, which greatly enhance the immunological functions of cancer patients[1,2]. Ekki-Youketsu-Fusei-Zai (EYFZ), one of the TCMs, is a mixture composed of six kinds of crude drugs, which have been used for cancer patients in China. Recent studies have shown that each crude drug has various biological activities. For example, *Astragali radix* has immunologically enhancing and anti-tumor effects[3-5], while *Angelicae radix* has anti-inflammatory, analgesic, interferon inducing, and immunopotentiating effects[6-9]. *Cervi parvum cornu* is known to show anti-tumor and immuno-enhancing effects by inhibiting the monoamine oxidase (MAO) activity[10-12], and *Zizyphi fructus* augments the function of natural killer cell as well as ciliary motility in the airway[13,14]. Finally, *Rehmanniae radix* is reported to have anti-tumor activity, enhance cytotoxic T lymphocytes (CTL) activity and induce IL-2 production by T cells[15,16].

医学博士　乙第792号　平成13年12月21日 (埼玉医科大学)

Macrophages are known to be activated *in vitro* by a variety of agents, leading to an increase of metabolism, syntheses and release of lysosomal enzyme, phagocytosis, and anti-mitotic or anti-tumor effects. Many of these agents are generally potent immunological adjuvants or immunopotentiators, some of which are natural products called biological response modifiers (BRM)[17] and possess functions affecting immune system and host defense mechanisms. BRM also alters the host biological responses[18], and is effective in suppressing tumor cells[19]. Among these products, bacterial lipopolysaccharides (LPS) is one of the strongest activators of macrophage[20,21]. To verify the mechanisms underlying the LPS action on macrophage, murine macrophage-like cell line, J774.1 has been so far used in many investigations[22-30].

Though each component of EYFZ has shown various biological activities and EYFZ has been used for cancer patients, its anti-tumor effects and immuno-enhancing effects have not been investigated. In this study, we examined the effects of oral administration of EYFZ on the survival, tumor size, body weight and natural killer (NK) cell activity of tumor-bearing mice *in vivo* and also examined the effect of EYFZ on cytokine production of J774.1 *in vitro* in order to elucidate the relationship between immuno-enhancing effects of EYFZ and its anti-tumor effects.

Materials and Methods

Animals and tumor cell line Specific-pathogen-free BALB/c female mice at the age of 5 weeks, originally purchased from Japan CLEA Co, were used throughout this experiment and approved by institutional animal care committee. Colon-26 cell line, colon-adenocarcinoma from BALB/c mice, was generously provided by Prof. Kikuo Nomoto, Kyushu University, and cultured *in vitro* with the basal medium (RPMI1640 with 2.0 g/L $NaHCO_3$, 1.0 g/L HEPES, 0.6 g/L L-glutamine, and 0.25 mg/L Kanamycin) containing 10 % fetal bovine serum (FBS). By a strong pipetting procedure, we obtained a single cell suspension and 5×10^5 cells were implanted subcutaneously into the back of mouse. Murine lymphoma YAC-1 cell was maintained in the same culture medium as shown above.

Preparation of EYFZ EYFZ is a mixture composed of six kinds of crude drugs as shown in Table 1. All of the crude drugs were obtained from Tochimoto, Ltd., Osaka, and EYFZ was prepared as follows. First, a mixture of *Astragali radix* (10.0 g), *Zizyphy fructus* (5.0 g), *Amomi semen* (5.0 g), *Angelica radix* (8.0 g), *Cervi parvum cornu* (5.0 g), and *Rehmanniae radix* (10.0 g) was added to 200 ml distilled water (DW) and soaked for 20 min at room temperature. Then, it was boiled for 30 min for extraction of effective substances, and the solution was centrifuged at 3000 rpm for 20 min. Finally, the supernatant was condensed to 43 ml (1 g crude drugs/ml) by heating evaporation, and diluted with DW to desired concentrations for *in vivo* and *in vitro* experiments, and orally administered to mice at a dose of 716.7 mg crude drugs/kg. This dose was considered appropriate because oral administration of EYFZ into human is traditionally 43 g crude drugs/60 kg/day.

Evaluation of survival, tumor size, and body weight of tumor-bearing mice Colon-26 (5×10^5) cells were subcutaneously implanted into 6-week-old mouse at once, and simultaneously the oral administration of EYFZ was started. Control mice received the same volume of saline instead of EYFZ. Thereafter, the survival of these mice was monitored everyday for evaluating the life-prolonging effect. The tumor size and body weight were examined 2-3 times a week. The major axis (a) and minor axis (b) of the tumor were measured, and then size was estimated by using the formula $ab^2/2$[31].

Assay of splenic NK cell activity The splenic NK cell activity of tumor-bearing mice was determined by lactate dehydrogenase (LDH) assay[32,33]. The spleen cell suspension was prepared by squeezing the spleen between two glass slides. The distilled water was added to the spleen cell suspension for provoking lysis of red blood cells. After washing three times with serum-free basal medium, the cells were incubated in a 25 cm^2 culture flask (FALCON, Becton Dickinson) in serum-free basal medium at 37 ℃ in a 5 % CO_2 incubator for 2 hr to remove adherent cells. The non-adherent cells were collected as effector cells. Effector cells (5×10^6 cells/ml) were incubated with NK-sensitive target cells, YAC-1 (5×10^4 cells/ml), in a total volume of 0.2 ml/well using 96 well round bottomed microplate (IWAKI Glass Co, Ltd.). An effector-target ratio of 100:1 was considered optimum. The plate was incubated for 4 hr at 37 ℃ in a 5 % CO_2 incubator. After incubation, 0.05 ml of the supernatant from each well was collected, then used for LDH assay to determine the cytotoxic activity using the LDH cytotoxic Kit (Wako Pure Chemical Industries, Ltd.). The percentage of specific release was calculated according to the following formula: % specific lytic activity = (experiment

Table 1. The Botanical Origins of Crube Drugns of "Ekki-Youketsu-Fusei-Zai"

Crude drug	Botanical origin (Family name)	Harvesting time	Representative defined compounds	Ratio
Astragali Radix	* *Astragalus membranaceus* Bge. Var. *Ongholicus* (Bge.) *mongholicus* (Bge.) Hsiao (A. *mongholicus* Bge.) * *Astragalus membranaceus* (Fisch.) Bge.	spring or autumn	β-sitosterol, D-β-Asparagine, disaccharide, 2'-4'-dihydroxy-5,6-dimethoxy-isoflavane, calycosin, formononetin, astragaloside Ⅰ.Ⅱ.Ⅲ.Ⅳ	10.0 g
Zizyphi Fructus	**Ziziphus jujubc* (Rhamnaceae)	autumn	Sugar, phlegm, malic acid, tartaric acid	5.0g
Amomi Semen	* *Amomum villosum* Lour. * *Amomum Xanthioides* Wall.	autumn	Borneol, dextrogyric camphor, bornyl acetate, linalool, nerolide	5.0g
Angelicae Radix	* *Angelic sinensis* (Oliv.) Diels	autumn	Ligustilide, n-butylidene-phthalide, sesquiterpenes A.B, carvacrol	8.0g
Cervi Parvum Cornu	* *Cervus nippon* Temminck * *Cervus elaphus* L.	spring or autumn	Ceramide, oestrone, 17-β-Estradiol	5.0g
Rehmanniae Radix	* *Rehmannia glutinosa* Libosch.	autumn	Catalpol, stachyose, amino acid, β-Sitosterol	10.0g

Medicine was prepared by blending the crude drugs in the ratios indicated above

release - spontaneous release) / (maximum release - spontaneous release) ×100

J774.1 cell line culture The murine macrophage-like cell line, J774.1, was obtained from Riken Cell Bank (RCB, Japan). The cells were maintained in 75 cm^2 plastic culture flasks (Falcon) in basal medium containing 5 % fetal bovine serum. Cells were incubated at 37℃ in a humidified atmosphere containing 5 % CO_2. Cells harvested by gentle scraping were passaged every 3-4 days by diluting 1: 10 with fresh medium.

RT-PCR J774.1 cells were suspended in culture medium at a cell concentration of 5×10^4 cells/ml, and 5 ml of the cell suspension was plated in a 25 cm^2 plastic culture flask (Falcon). Cells were cultured for 24 and 48 hrs in the presence of 1.2 mg crude drugs/ml of EYFZ, 10 ng/ml LPS or culture medium only, respectively, and after incubation, cells were harvested by scraping and quickly frozen and stored at −80 ℃ . Total cellular RNAs were extracted from 1 x 10^6 J774.1 cells by the use of Rneasy Mini Kit (QIAGEN). The first strand cDNA synthesis was performed by incubating 0.5μg of either RNA sample in a total reaction volume of 20μl containing 2μl of 10 mM dNTP mixture, 2μl of 10 mM oligo-(dT), 10X reaction buffer for AMV reverse transcriptase, and 4 units (U) of AMV reverse transcripase XL (TOYOBO, Japan) at 42 ℃ for 1hr. The sequence of oligo-(dT) was 5'-GCTCTAGATT TTTTTTTTTTTTTTTTTTTT-3'. Two μl of the first strand synthesis production and 2μl of each of 10 mM oligonucleotide primers were added to the reaction mixture (100μl) containing 8μl of 10 mM dNTP mixture, 8μl of 25 mM $MgCl_2$, 10μl 10X Ex TaqTM buffer, and 4 units (U) of Ex TaqTM (TAKARA, Japan). Each reaction was carried out as follows: IL-1β; denaturing at 94 ℃ for 5 min, 30 cycles of amplification (94 ℃ for 1 min, 58 ℃ for 2 min, and 72 ℃ for 3 min) and extension at 72 ℃ for 10 min. IL-12p40 and IFN-γ; denaturing at 94 ℃ for 5 min, 30 cycles of amplification (94 ℃ for 40 sec, 60 ℃ for 20 sec, and 72 ℃ for 49 sec) and extension at 72 ℃ for 5 min. IL-12p35, TNF-α and β-actin; denaturing at 94 ℃ for 5 min, 30 cycles of amplification (94 ℃ for 1 min, 55 ℃ for 1 min, and 72 ℃ for 2 min) and extension at 72 ℃ for 7 min.

Measurements of IL-12 IL-12 production by J774.1 cells was assayed by using ELISA kits OptEIATM Mouse IL-12 (p70) Set (PHARMINGEN) according to the protocols by manufacturer. Cells were cultured for 24, 48 and 72 hrs in the presence of 0.15 mg/ml, 1.2 mg/ml and 3.6 mg crude drugs/ml of EYFZ, 10 ng/ml LPS or culture medium only, respectively, and after incubation, the culture supernatants were collected and stored at −80℃ until use.

Statistical analysis Survival curve was determined using the method of Kaplan and Meier, and the log rank test was used to calculate the significance. Other data were statistically analyzed based on the Student's t test, and the differences were recognized significant with p value less than 0.05. The results were expressed as mean ± standard deviation (SD).

Results

Effect of EYFZ on a life-prolongation, tumor size and the body weight of tumor bearing mice We first examined the effect of oral administration of EYFZ on the survival of tumor-bearing mice. When EYFZ (716.7 mg crude drugs/kg/day) was continuously administrated to the mice which had been implanted subcutaneously with colon-26 for 28 days, the

life-prolonging effect was found as shown in Fig.1. All of tumor-bearing mice in the control group that received only the saline died within 59 days after the onset of this experiment. On the other hand, the tumor-bearing mice treated with EYFZ showed a significant life-prolonging effect as compared to the control ($p<0.01$), and died in 70 days on average.

Second, we compared the tumor size of EYFZ-administrated group mice with that of control group mice. The tumor size, as described in Materials and Methods, was calculated by measuring the major (a) and minor (b) axis of formed tumor tissue based on the formula $ab^2/2$. Result obtained from observation for 34 days revealed that the tumor size in EYFZ-administrated mice was smaller than that in the control mice (Fig.2). Although the tumor size on day-13 was almost similar to that in the control, those on day-20 and on day-27 were clearly smaller than that in the control group ($p<0.5$ and $p<0.1$, respectively).

Third, we examined the effect of oral administration of EYFZ on the body weight in tumor-bearing mice. When we observed the body weight of mice in the EYFZ-treated or control mice successively for 28 days after the onset of this experiment, it was shown that the body weight in the EYFZ-treated mice was much larger than that in the control mice (Fig.3). Particularly, the body weight in the treated mice was significantly larger than that in the control mice on day-14 ($p<0.05$).

Effect of EYFZ on NK cell activity of tumor-bearing mice We examined the effect of oral administration of EYFZ on splenic NK cell cytotoxicity in tumor-bearing mice. We found that the NK cytotoxic activity of splenic cells in orally EYFZ-administrated mice was significantly higher than that in control mice on the day-17 and day-24 after the onset of transplantation and oral administration, as shown in Fig. 4 ($p<0.01$).

Effect of EYFZ on the expression of cytokine mRNAs in J774.1 cells The cytokine mRNA expression of J774.1 cells treated with or without EYFZ was investigated. The gel electrophoretic patterns of the RT-PCR products of IL-1β, IL-12p35, IL-12p40, IFN-γ, TNF-α and β-actin are presented in Fig. 5. The expression level of IL-12p35 and IL-12p40 was induced in J774.1 cells after 12hrs and 24hrs of the treatment

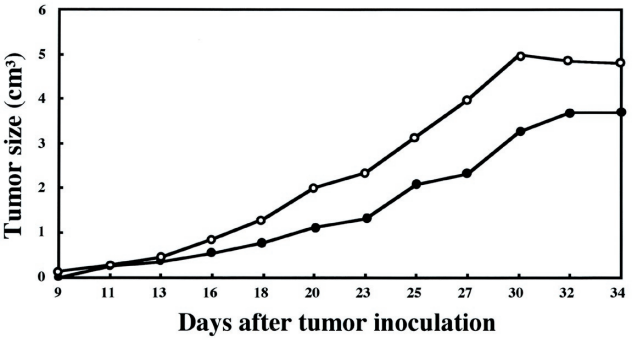

Fig. 2. Effect of Ekki-Youketsu-Fusei-Zai (EYFZ) on the *in vivo* growth of colon-26 tumor cells. Mice treated with EYFZ (716.7mg crude drugs/kg) and control mice without EYFZ were inoculated with colon-26 cells according to the same protocol as in Fig. 1. Tumor growth was measured after implantation and calculated as shown in Material and Methods. The tumor size was measured from day-9 to day-34 after transplantation. (●): treatment group mice, (○): control group mice.

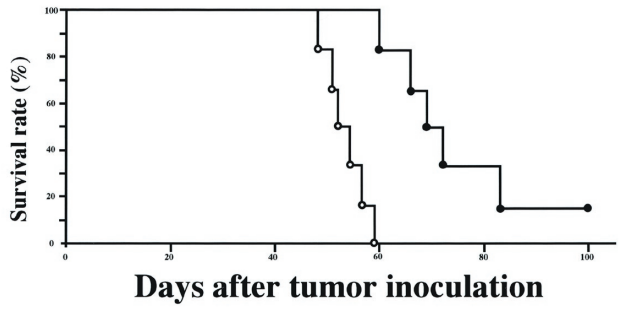

Fig. 1. Effect of Ekki-Youketsu-Fusei-Zai (EYFZ) on survival of mice inoculated with colon-26 cells. Six BALB/c female mice per group were inoculated s.c with colon-26 ($5x10^5$cells/mouse). Treatment group mice were orally administered with EYFZ (716.7mg crude drugs/kg) for 28 days just after the subcutaneous implantation of colon-26 cell line. The control group mice received only saline. Their survival rates are shown. (●): treatment group mice, (○): control group mice. $P<0.01$; by log rank test.

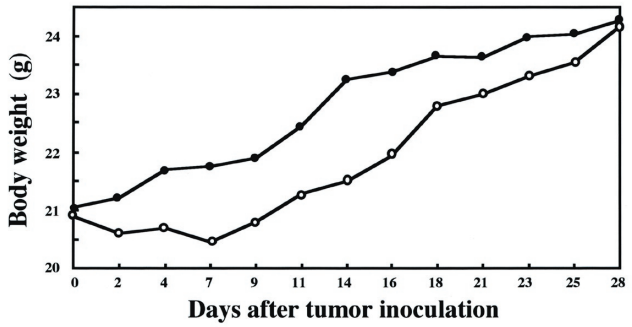

Fig. 3. Effect of Ekki-Youketsu-Fusei-Zai (EYFZ) on body weight loss of mice inoculated with colon-26 cells. Mice treated with EYFZ (716.7mg crude drugs/kg) and control mice without EYFZ were inoculated with colon-26 cells according to the same protocol as in Fig. 1. After implantation, the body weight was measured 2 or 3 times a week and finally on day-28. (●): treatment group mice, (○): control group mice.

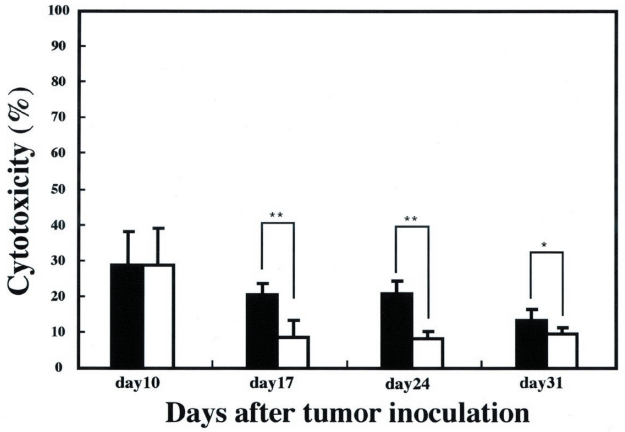

Fig. 4. Effect of Ekki-Youketsu-Fusei-Zai (EYFZ) on NK cell activity of mice inoculated with colon-26 cells. Five BALB/c female mice per group were inoculated s.c with colon-26 cells (5×10^5 cells/mouse). Treatment group mice were orally administered with EYFZ (716.7mg crude drugs/kg) for 28 days just after the subcutaneous implantation of colon-26 cell line. Control group mice received only saline. Each column and vertical bar represents the mean±standard deviation of 5 mice on day-10, day-17, day-24 and day-31 after inoculation. (■): treatment group mice, (□): control group mice. *P＜0.5 and **P＜0.01; by Student's two-tailed test.

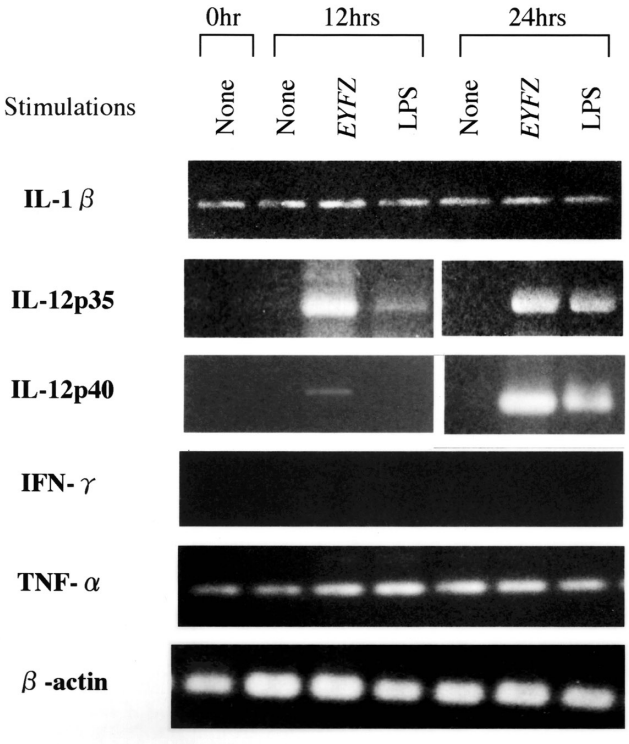

Fig. 5. Effects of EYFZ on the expression of cytokine mRNAs. J774.1 stimulated with EYFZ or LPS in culture were assayed for IL-1 β , IL 12p35, IL-12p40, IFN- γ , TNF- α and β -actin mRNA by RT-PCR. The results are for a typical example of repeated experiments.

with EYFZ, though the expression of β-actin, a house keeping gene, was almost constant in each sample. We could not find any differences of other cytokine expressions between treated and untreated cells.

Effect of EYFZ on cytokine production in J774.1 cells We next examined whether or not this increased expression of IL-12 induced by EYFZ occurs at the protein level. After J774.1 cells were incubated with 0.15 mg/ml, 1.2 mg/ml and 3.6 mg crude drugs/ml of EYFZ for 24, 48 and 72 hrs, IL-12 in the culture supernatants was assayed by ELISA. As shown in Fig. 6, IL-12 secreted from J774.1 cells stimulated with EYFZ in the culture supernatants was significantly higher in concentration compared to those of the control.

Discussion

In this experiment, we examined whether or not EYFZ, one of the TCMs, affected the anti-tumor activity in mice into which the murine colon-26 carcinoma cell line was subcutaneously implanted. This colon-26 cell line, an undifferentiated carcinoma induced by the carcinogen N-nitroso-N-methylurethan, has been successfully used as the model of tumor-bearing mice and cachexia[31,34]. In our study, colon-26 cells could grow well after subcutaneous implantation into the normal BALB/c mice. Oral administration of EYFZ caused a statistically significant prolonging effect on survival in tumor-bearing mice as compared with the control mice. We also examined both the tumor size and body weight. The tumor size in EYFZ-administrated mice was shown to be much more decreased than that in the control mice. Particularly, the tumor size in the treated mice after 27 days became significantly smaller than that in the control. Hence, it is suggested that the oral administration of EYFZ was more effective compared with oral administration of saline for both the survival and the decrease of a tumor size in tumor-bearing mice.

The cachexia, an exhaustive state with severe weight loss, is a serious problem in cancer patients affecting their morbidity and mortality. It lowers their quality of life and shortens their life-span[35,36]. Colon-26 cell line has been successfully used as the model of such cachexia by tumor growth[31]. Thus, we observed the body weight of tumor-bearing mice everyday following the subcutaneous implantation of colon-26 cells into both the EYFZ-treated and the control mice. Results clearly showed that oral administration of EYFZ led to the better increase of body weight as compared to the control without EYFZ. Though the body weight of the control mice was initially lower than that of

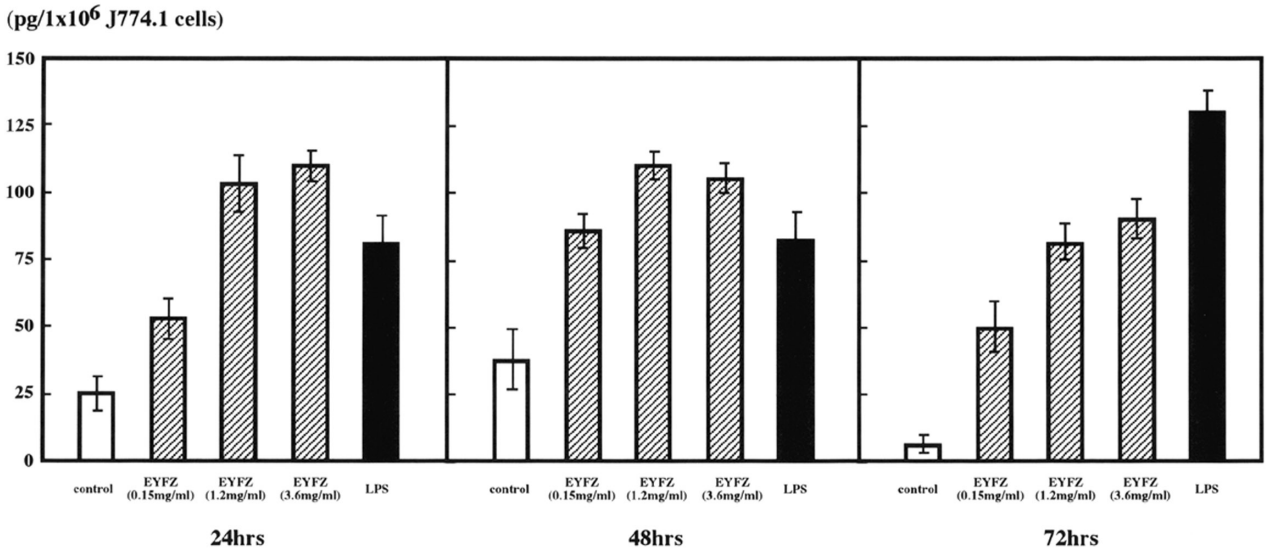

Fig. 6 The secretion of IL-12 from J774.1 stimulated with EYFZ.The secretion of IL-12 from J774.1 after incubation for 24, 48, and 72 hrs. J774.1 cells (1×10^6 cells/ml) were cultured in the presence of EYFZ (0.15 mg/ml, 1.2 mg/ml and 3.6 mg crude drugs/ml) (hatched bars), LPS (10 ng/ml) (solid bars), or control (culture medium only). Values represent the mean ± S.D. of four independent experiments.

the EYFZ-treated mice, it finally reached the similar level to that of the EYFZ-treated mice. This means the tumor growth in the control mice leading to the increase of body weight. We think the difference of the body weight between the two groups of mice on day-14 is much more important than that on the later days, and it reflects the improvement of cachexia by EYFZ. However, since there was a possibility that EYFZ influenced the increase of the body weight of cancer-bearing mice from the nutritious viewpoints, it will be necessary to further examine the effect of oral-administration of EYFZ on body weight of the normal mice without cancer cell in the future.

NK cells exhibit spontaneous cytotoxic activity in a non-major histocompatibilty complex (MHC) restricted manner against virus-infected cells and cancer cells *in vivo* and their activity can be augmented by administration of interferon-γ (IFN-γ)[35-38]. Some papers showed that some kinds of crude drugs and TCMs exert anti-tumor effects by activation of NK cells[13,39-43]. Therefore, we also tried to examine the cytotoxic effect of oral administration of EYFZ on splenic NK cell activity in tumor-bearing mice on day-10, day-17, day-24 and day-31 following the subcutaneous implantation of colon-26 cells. Results clearly showed that the NK activity of spleen cells in orally EYFZ-administrated mice was significantly higher than that in control mice on the day-17 and day-24 after transplantation and oral EYFZ administration. In our present experiment, it was hard to investigate the cytokine production of NK cells in the spleen due to the insufficient number of splenic NK cells. However, as the NK activity was clearly augmented in the EYFZ-treated mice, further evaluation for EYFZ in anti-tumor activity seems to be needed by examining the effect of oral-administration of EYFZ on the cytokine production of NK cells in the spleen.

EYFZ does not show the direct cytotoxicity on some tumor cell lines including colon-26, A549 and Kato III (data not shown). This greatly suggests that the efficacy of EYFZ on survival and tumor-growth in tumor-bearing mice is attributable to the enhancement of host defense or immune system such as NK cell activity but not to the direct cytotoxicity against tumor cells.

To elucidate the relationship between the macrophage and anti-tumor effects of EYFZ, we also tried to examine the effects of oral administration of EYFZ on the functions of peritoneal macrophage in tumor-bearing BALB/c mice; however, it was difficult to get the some effects of EYFZ on macrophage functions. First, for example, mice got weak after the implantation of colon-26 cell, and it was difficult to collect enough cells of resting macrophage without thioglycollate-stimulation. Second, since the thioglycollate itself strongly stimulated the resting peritoneal macrophage in tumor-bearing mice, we could not find any difference between the EYFZ-administrated and control mice on functions of peritoneal macrophage. Third, J774.1 macrophage-like cell line was originated from the BALB/c mice and can be used for the study of macrophage function *in vitro* because this cell line

was reported to show the same cytokine production as the macrophages[44-47]. Thus, in the present study, we examined the effect of EYFZ on cytokine production of J774.1 *in vitro* to elucidate the mechanisms underlying the EYFZ action on immune responses.

Macrophages are involved in almost all stages of the immune responses and play a role in the initial response to microbial infection before T- and B cell immunity are evoked. Mechanisms by which macrophages act as effector cells in a host defense system include both intracellular and extracellular cytokine secretion activities. Thus, we investigated here whether or not EYFZ could stimulate the expression and the secretion of cytokine from J774.1.

As shown in this study, EYFZ was able to induce IL-12 expression and production by J774.1. IL-12 has recently been brought into focus as an effector molecule to enhance immunity by murine macrophages[48-50]. Production of IL-12 by macrophages can be induced by interaction with activated T cells, which provides costimulatory signals via molecules such as CD40 ligand. These signals appear to be essential, because their inhibition can abrogate IL-12 production. IL-12 exerts multiple effects on T and NK cells including the augmentation of IFN-γ production, proliferation, and cytotoxic activity, and also plays an important role to determine a Th1/Th2 balance[51]. IL-12 is a heterodimeric cytokine composed of disulfide linked p40 and p35 subunits; both subunits have to be expressed within the same cell to produce biologically active p70 heterodimer[52]. It has been shown that p40 mRNA expression is up-regulated in the cells producing IL-12, whereas p35 mRNA is constitutively expressed in various cells[53]. As shown in Fig.4, the expressions of IL-12p35 and IL-12p40 were induced similarly as assessed at 12hrs and 24hrs after the treatment with EYFZ. Moreover, our results of ELISA show that this increased expression of IL-12p35 and IL-12p40 induced by EYFZ occurs at the protein level.

It was reported that IL-12 has anti-tumor effect[54-57] and a powerful anti-tumor activity in mice against 17 different lines of transplantable murine tumors, including carcinomas, sarcomas, melanomas and lymphomas[58]. IL-12 is much more effective in anti-tumor effect than other cytokines such as IL-2 and IFN-α, and is effective at the doses with much lower toxicity. In our study, relationship is unclear between the effects of EYFZ on anti-tumor and enhancement of NK function *in vivo* and activation of macrophage-like cell line J774.1 *in vitro*. However, some papers showed that components of crude drugs of EYFZ include polysaccharide[59-64], which can activate the function of macrophage. Thus, whether or not oral administration of EYFZ can induce macrophages to produce endogenous IL-12 is not clear now. So, this elucidation needs more study in the future.

In conclusion, we have found that EYFZ has the anti-tumor effects on colon-26 implanted mice via augmentation of NK cell activity and markedly activates J774.1 to produce IL-12, which is potentially an immune enhancer. Therefore, it would be important to investigate further the effective mechanism of EYFZ on the immune system.

Acknowledgement

I would like to thank Drs. Prof. Toshitaka Akatsuka and Prof. Haruhisa Wago for their kind help and valuable suggestions in this experiment and Ms Kaori Nakajima and Ms Xinling Ma for kind assistance with cell culture and mice-rearing. I also appreciate Dr. Kenichiro Hasumi for critical reading of this manuscript and financial help in this investigation.

References

1) Adachi I, Watanabe T. Role of supporting therapy of Juzentaiho-to (JTT) in advanced breast cancer patients. Gan To Kagaku Ryoho 1989;16:1538-43. (in Japanese)
2) Lee YS, Chung IS, Lee IR, Kin KH, Hong WS, Yun YS. Activation of multiple effector pathways of immune system by the antineoplastic immunostimulator acidic polysaccharide ginsan isolated from Panax ginseng. Anticancer Res 1997;17:323-31.
3) Zhao KS, Mancini C, Doria G. Enhancement of the immune response in mice by Astragalus membranaceus extracts. Immunopharmacology 1990;20:225-33.
4) Yoshida Y, Wang MQ, Liu JN, Shan BE,Yamashita U. Immunomodulating activity of Chinese medicinal herbs and Oldenlandia diffusa in particular. Int J Immunopharmacol 1997;19:359-70.
5) Lau BH, Ruckle HC, Botolazzo T, Lui PD. Chinese medicinal herbs inhibit growth of murine renal cell carcinoma. Cancer Biother 1994;9:153-61.
6) Kojima S, Inaba K, Kobayashi S, Kimura M. Inhibitory effects of traditional Chinese medicine Shimotsu-to and its included crude fractions on adjuvant-induced chronic inflammation of mice. Biol Pharm Bull 1996;19:47-52.
7) Tanaka S, Ikeshiro Y, Tabata M, Konoshima M. Anti-

nociceptive substances from the roots of Angelica acutiloba. Arzneimittelforschung 1977;27:2039-45.
8) Kumazawa Y, Nakatsuru Y, Fujisawa H,Nishimura C, Mizunoe K,Otsuka Y,et al. Lymphocyte activation by a polysaccharide fraction separated from hot water extracts of Angelica acutiloba Kitagawa. J Pharmacobiodyn 1985;8:417-24.
9) Wang NL, Kiyohara H, Matsumoto T, Otsuka H, Hirano M, Yamada H. Polyclonal antibody against a complement-activating pectin from the roots of Angelica acutiloba. Planta Med 1994;60:425-9.
10) Wang BX, Zhao XH, Qi SB, Kaneko S, Hattori M, Namba T, et al. Effects of repeated administration of deer antler extract on biochemical changes related to aging in senescence-accelerated mice. Chem Pharm Bull(Tokyo) 1988;36:2587-92.
11) Wang BX, Liu AJ, Wang QG, Wei GR, Chui JC, Yang N, et al. Study on pharmacology of polysaccharide of deer antler. Chinese Dispatches of Pharmacology 1985;3:9-12.(in Chinese)
12) Sun XB, Zhou CC. Effect of deer antle extract on immunological function. Chinese Patent Pedicine Research 1986;2:24-5.(in Chinese)
13) Yamaoka Y, Kawakita T, Kaneko M, Nomoto K. A polysaccharide fraction of *Zizyphi fructus* in augmenting natural killer activity by oral administration. Biol Pharm Bull 1996;19:936-9.
14) Tamaoki J, Kondo M, Tagaya E, Takemura K, Konno K. *Zizyphi fructus*, a constituent of antiasthmatic herbal medicine, stimulates airway epithelial ciliary motility through nitric oxide generation. Exp Lung Res 1996;22:255-66.
15) Hamada M, Fujii Y, Yamamoto H, Miyazawa Y, Shui SM, Tung YC, et al. Effect of a kanpo medicine, zyuzentaihoto, on the immune reactivity of tumor bearing mice. J Ethnopharmacol 1988;24:311-20.
16) Chen LZ, Feng XW, Zhou JH. Effects of Rehmannia glutinosa polysaccharide b on T-lymphocytes in mice bearing sarcoma 180. Chung Kuo Yao Li Hsuch Pao 1995;16:337-40. (in Chinese)
17) Johnson WJ, Adams DO, A Volkman. Mononuclear phagocyte biology. Marcel Dekker, Inc., New York, 1984:279-300.
18) Oldham RK. Biological Response Modifiers Programme and cancer chemotherapy. Int J Tissue React 1982;4(3):173-88.
19) Oldham RK. Biological response modifiers. J Natl Cancer Inst 1983;70(5):789-96.
20) Alexander P, Evans R.Endotoxin and double stranded RNA render macrophages cytotoxic. Nat New Biol. 1971;232(29):76-8.
21) Johnston RB Jr, Godzik CA, Cohn ZA. Increased superoxide anion production by immunologically activated and chemically elicited macrophages. J Exp Med 1978;148(1):115-27.
22) Amano F, Akamatsu Y. A lipopolysaccharide (LPS)-resistant mutant isolated from a macrophagelike cell line, J774.1, exhibits an altered activated-macrophage phenotype in response to LPS. Infect Immun 1991;59(6):2166-74.
23) Amano F, Nishijima M, Akamatsu Y. A monosaccharide precursor of Escherichia coli lipid A has the ability to induce tumor-cytotoxic factor production by a murine macrophage-like cell line, J774.1. J Immunol 1986;136(11):4122-7.
24) Noda T, Amano F. Defect in the induction of nitric oxide synthase by lipopolysaccharide (LPS) in an LPS-resistant mutant of a murine macrophage-like cell line, J774.1. Biol Pharm Bull 1998;21(7):673-7.
25) Salvemini D, Pistelli A, Mollace V, Anggard E, Vane J. The metabolism of glyceryl trinitrate to nitric oxide in the macrophage cell line J774 and its induction by Escherichia coli lipopolysaccharide. Biochem Pharmacol 1992;44(1):17-24.
26) Kura F, Suzuki K, Watanabe H, Akamatsu Y, Amano F. Difference in Legionella pneumophila growth permissiveness between J774.1 murine macrophage-like JA-4 cells and lipopolysaccharide (LPS)-resistant mutant cells, LPS1916, after stimulation with LPS. Infect Immun 1994;62(12):5419-23.
27) Inoue S, Itagaki S, Amano F. Intracellular killing of Listeria monocytogenes in the J774.1 macrophage-like cell line and the lipopolysaccharide (LPS)-resistant mutant LPS1916 cell line defective in the generation of reactive oxygen intermediates after LPS treatment. Infect Immun 1995;63(5):1876-86.
28) Fujihara M, Muroi M, Muroi Y, Ito N, Suzuki T. Mechanism of lipopolysaccharide-triggered junB activation in a mouse macrophage-like cell line (J774). J Biol Chem 1993;268(20):14898-905.
29) Nishijima M, Hara-Kuge S, Takasuka N, Akagawa K, Setouchi M, Matsuura K, et al. Identification of a biochemical lesion, and characteristic response to lipopolysaccharide (LPS) of a cultured macrophage-like cell mutant with defective LPS-binding. J Biochem (Tokyo) 1994;116(5):1082-7.
30) Suda Y, Kirikae T, Shiyama T,Yasukochi T, Kirikae F, Nakano M, et al. Macrophage activation in response to S-form lipopolysaccharides (LPS) separated by centrifugal partition chromatography from wild-type

LPS: effects of the O polysaccharide portion of LPS. Biochem Biophys Res Commun 1995;210(3):678-85.
31) Tanaka Y, Eda H, Tanaka T, Udagawa T, Ishikawa T, Horii I, et al. Experimental cancer cachexia induced by transplantable colon-26 adenocarcinoma in mice. Cancer Res 1990;50:2290-5.
32) Korzeniewski C, Callewaert DM. An enzyme-release assay for natural cytotoxicity. J Immunol Methods 1983;64:313-20.
33) Decker T, Lohmann-Matthes ML. A quick and simple method for the quantitation of lactate dehydrogenase release in measurements of cellular cytotoxicity and tumor necrosis factor(TNF)activity. J Immunol Methods 1988;115:61-9.
34) Corbett TH,Griswold DP Jr,Roberts BJ,Peckham JC, Schabel FM Jr. Tumor induction relationships in development of transplantable cancers of the colon in mice for chemotherapy assays, with a note on carcinogen structure. Cancer Res 1975;35:2434-9.
35) DeWys W.D,Begg D,Lavin P.T,Band P.R, Bennett J.M, et al. Prognostic effect of weight loss prior to chemotherapy in cancer patients. Am. J.Med 1980;69:491-7.
36) Soda K, Kawakama M, Kashii A, Miyata M. Characterization of mice bearing subclones of colon-26 adenocarcinoma disqualifies interleukin-6 as the sole inducee of cachexia. Jpn. J. Cancer Res 1994;85:1124-30.
37) Trinchieri G. Biology of natural killer cells. Adv Immunol 1989;47:187-98.
38) Vujanovic NL,Basse P,Herberman RB,Whiteside TL. Anti-tumor functions of natural killer cells and control of metastases. Method Companion Methods Enzymol 1996;9:394.
39) Biron CA. Activation and function of natural killer cells responses duing viral infections. Curr Opin Immunol 1997;9:24.
40) Kaneko M, Kawakita T, Tauchi Y, Sato Y, Suzuki A, Nomoto K. Augmentation of NK activity after oral administration of a Traditional Chinese Medicine, Xiao-Chai-Hu-Tang (ShoSaiKo-To). Immumopharmacology and Immunotoxicology 1994;16:41-5.
41) Kurashige S,Jin R,Akuzawa T,Endo F. Anticarcinogenic effects of shikaron, a preparation of eight Chinese herbs in mice treated with a carcinogen, N-buty-N' butanolnitrosoamine. Cancer Invest 1998;16:166-9.
42) Cho JM, Sato N, Kikuchi K. Prophylactic anti-tumor effect of Hochu-ekki-to (TJ41) by enhanceing natural killer cell activity. *In vivo* 1991;5:389-91.
43) Zee-cheng RK. Shi-quan-da-bu-tang (ten significant tonic decoction), SQT. A potent Chinese biological response modifier in cancer immunotherapy, potentiation and detoxification of anticancer durgs. Method Find Exp Clin Pharmacol 1992;14:725-36.
44) Klostergaard J, Foster WA, Leroux ME. Tumoricidal effet mechanisms of murine BCG-activated macrophages. I. Parameters of production and initial characterization of a cytolytic factor serologically related to necrosin. J Biol Response Mod 1987;6(3):313-30.
45) Nishikawa K, Arai H, Inoue K. Scavenger receptor-mediated uptake and metabolism of lipidvesicles containing acidic phospholipids by mouse peritoneal macrophages. J Biol Chem 1990;265(9):5226-31.
46) Kawakami K,Qureshi MH,Koguchi Y,Nakajima K, Saito A.Differential effect of cryptococcus neoformans on the production of IL-12p40 and IL-10 by murine macrophages stimulated with lipopolysaccharide and gamma interferon. FEMS Microbiol Lett 1999;175(1):87-94.
47) Lei MG, Morrison DC. Differential expression of caveolin-1 in lipopolysaccharide-activated murine macrophages. Infect Immun 2000;68(9):5084-9.
48) Kato T,Hakamada R,Yamane H,Nariuchi H. Induction of IL-12 p40 messenger RNA expression and IL-12 production of macrophages via CD40-CD40 ligand interaction. J Immunol 1996;156(10):3932-8.
49) Shu U,Kiniwa M,Wu CY,Maliszewski C,Vezzio N, Hakimi J,et al. Activated T cells induce interleukin-12 production by monocytes via CD40-CD40 ligand interaction. Eur J Immunol 1995;25(4):1125-8.
50) Kato T, Yamane H, Nariuchi H. Differential effects of LPS and CD40 ligand stimulations on the induction of IL-12 production by dendritic cells and macrophages. Cell Immunol 1997;181(1):59-67.
51) Hsieh CS, Macatonia SE, Tripp CS, Wolf SF, O'Garra A, Murphy KM.Development of Th1 CD4+ T cells through IL-12 produced by Listeria-induced macrophages. Science 1993;260(5107):547-9.
52) Manetti R, Parronchi P, Giudizi MG, Piccinni MP, Maggi E, Trinchieri G, et al. Natural killer cell stimulatory factor (interleukin-12 [IL-12]) induces T helper type 1 (Th1)-specific immune responses and inhibits the development of IL-4 producing Th cells. J Exp Med 1993;177(4):1199-204.
53) Seder RA, Gazzinelli R, Sher A, Paul WE. Interleukin-12 acts directly on $CD4^{+}$T cells to enhance priming for interferon gamma production and diminishes interleukin-4 inhibition

of such priming. Proc Natl Acad Sci U S A 1993;90(21):10188-92.
54) Roy EJ, Gawlick U, Orr BA, Rund LA, Webb AG, Kranz DM. IL-12 treatment of endogenously arising murine brain tumors. J Immunol 2000;165(12):7293-9.
55) Smyth MJ, Taniguchi M, Street SE. The anti-tumor activity of IL-12: mechanisms of innate immunity that are model and dose dependent. J Immunol 2000;165(5):2665-70.
56) Takeda K, Hayakawa Y, Atsuta M, Hong S, Van Kaer L, Kobayashi K, et al. Relative contribution of NK and NKT cells to the anti-metastatic activities of IL-12. Int Immunol 2000;12(6):909-14.
57) Wang J, Nakamoto T, Kasaoka Y, Usui T, Hamada H. Anti-tumor effect of murine renal cell carcinoma cells genetically modified to express B7-1 combined with cytokine secreting fibroblasts. Hiroshima J Med Sci 2000;49(1):73-82.
58) Brunda MJ, Luistro L, Warrier RR, Wright RB, Hubbard BR, Murphy M, et al. Anti-tumor and anti-metastatic activity of interleukin-12 against murine tumors. J Exp Med 1993;178(4):1223-30.
59) Wang J, Ito H, Shimura K. Enhancing effect of antitumor polysaccharide from Astragalus or Radix hedysarum on C3 cleavage production of macrophages in mice. Jpn J Pharmacol 1989; 51(3):432-4.
60) Shimizu N, Tomoda M, Kanari M, Gonda R. An acidic polysaccharide having activity on the reticuloendothelial system from the root of Astragalus mongholicus. Chem Pharm Bull (Tokyo) 1991;39(11):2969-72.
61) Chen LZ, Feng XW, Zhou JH. Effects of Rehmannia glutinosa polysaccharide b on T-lymphocytes in mice bearing sarcoma 180. Zhongguo Yao Li Xue Bao 1995;16(4):337-40.
62) Kumazawa Y, Mizunoe K, Otsuka Y. Immunostimulating polysaccharide separated from hot water extract of Angelica acutiloba Kitagawa (Yamato tohki). Immunology 1982;47(1):75-83.
63) Ohno N, Matsumoto S, Suzuki I, Miyazaki T, Kumazawa Y, Otsuka Y, et al. Biochemical and physicochemical characterization of a mitogen obtained from an oriental crude drug, Tohki (Angelica actiloba Kitagawa). J Pharmacobiodyn 1983; 6(12):903-12.
64) Yamada H, Kiyohara H, Cyong JC, Kojima Y, Kumazawa Y, Otsuka Y. Studies on polysaccharides from Angelica acutiloba. Part 1. Fractionation and biological properties of polysaccharides. Planta Med 1984;50(2):163-7.

中国の伝統的漢方薬「益気養血扶正剤」の担癌マウスにおける延命とNK細胞機能に及ぼす影響並びにマウスのマクロファージJ774.1細胞のサイトカイン産生に対する影響

鄧　鴻
埼玉医科大学微生物学教室
（指導：赤塚　俊隆教授）

中国の伝統的漢方薬である「益気養血扶正剤」（以下EYFZと略す）の抗腫瘍活性と免疫細胞機能に及ぼす影響を知る目的で，第1にcolon-26腫瘍細胞株を移植した担癌マウスを用いて，28日間のEYFZ経口投与が担癌マウスの延命とNK細胞機能にいかなる影響を与えるかについて追究した．実験の結果，EYFZを連続的に投与された担癌マウスの寿命は非投与群と比較して有意に延命し，腫瘍サイズと体重の減少が抑制されることがわかった．一方，担癌マウスの脾臓細胞を採取して，その中のNK活性をYAC-1細胞への細胞傷害活性アッセイによって調べた結果，EYFZを投与された担癌マウスでは，非投与群と比較して有意にNK活性が高まることが判明した．第2にマクロファージ機能への影響を知る目的でマクロファージ細胞株J774.1細胞を用いてEYFZの影響を*in vitro* で追究した．J774.1の培養液に種々の濃度のEYFZを添加し，マクロファージ産生サイトカインへの影響をRT-PCR法及びELISA法によって調べた．実験の結果，RT-PCR法でサイトカインmRNA発現を調べると，特にIL-12p35及びIL-12p40がEYFZによって強く誘導された．またJ774.1の培養液中IL-12産生をELISA法で検討した結果，実際にIL-12がEYFZ存在下で強く産生されることがわかった．

以上の結果から，EYFZにはcolon-26を移植された担癌マウスの延命を引き起こす抗腫瘍効果があり，その作用機構の一つにNK活性の亢進とマクロファージ機能の活性化のあることが，強く示唆された．