Original

Effects of Traditional Chinese Medicine, Ekki-Youketsu-Fusei-Zai, on Human Peripheral Blood Lymphocytes *In Vitro*

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Abstract: The effect of Traditional Chinese Medicine (TCM), Ekki-Youketsu-Fusei-Zai (EYFZ), on human peripheral blood lymphocytes (PBL) was investigated *in vitro*. MTT assay showed that EYFZ could directly stimulate PBL to proliferate without mitogen. In addition, RT-PCR and ELISA revealed that though EYFZ induced the expression of interferon gamma (INF-γ) mRNA and the production of INF-γ in culture supernatant, it did not induce the experssion or the production of interleukin-2 (IL-2) or interleukin-4 (IL-4). Moreover, we also examined which kind of immunocyte was involved in the INF-γ production by EYFZ in culture supernatant by inhibition experiment with monoclonal antibodies. It was shown that the EYFZ induced the production of INF-γ by T cell subsets of $CD4^+$ and $CD8^+$ cells. Thus, these results suggest that EYFZ has some significant stimulating effects on proliferation of PBL and the induction of INF-γ by T cells subsets in human.

Keywords: Traditional Chinese Medicine (TCM), Ekki-Youketsu-Fusei-Zai (EYFZ), peripheral blood lymphocytes (PBL), proliferation, interferon-γ (INF-γ)
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Introduction

Many investigators have reported the efficacy of Traditional Chinese Medicine (TCM) through various pharmacological and biological studies, using *in vivo* and *in vitro* systems[1-3]. Ekki-Youketsu-Fusei-Zai (EYFZ), one of the TCMs, is a mixture composed of six kinds of crude drugs, which have been used for enhancing the immunological functions of cancer patients in China. Our studies have shown that EYFZ has anti-tumor effects such as a life-prolonging effect, tumor-growth inhibition, increase of body weight, and enhancement of cytotoxicity of natural killer (NK) cells in tumor-bearing mice[4]. Also, our study showed that EYFZ could induce the morphological change, the expression and the production of interleukin-12 (IL-12) on macrophage-like cell line, J774.1[5].

However, the effects of EYFZ on human peripheral blood lymphocytes (PBL) have not been reported so far *in vitro*. Therefore, in this study, we first examined whether or not EYFZ could stimulate PBL to proliferate without mitogen, express cytokine mRNA such as interferon gamma (IFN-γ), interleukin-2 (IL-2) or interleukin-4 (IL-4) and produce them in culture supernatant. Then, we performed an inhibition experiment using both anti-CD4 and anti-CD8 monoclonal antibodies to determine the involvement of $CD4^+$ or $CD8^+$ T cells in cytokine production.

Materials and Methods

Preparation of EYFZ EYFZ is a mixture composed of six kinds of crude drugs, as reported before[4]. The crude drugs were obtained from Tochimoto., Ltd., Osaka, and EYFZ was prepared as follows: A mixture of *Astragali radix* (10.0 g), *Zizyphi fructus* (5.0 g), *Amomi semen* (5.0 g), *Angelicae radix* (8.0 g), *Cervi parvum cornu* (5.0 g) and *Rehmanniae radix* (10.0 g) was added to 200 ml water and soaked for 20 min. 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 drug/ml) by heating evaporation, and diluted with distilled water to desired concentrations.

Effect of EYFZ on proliferation of human PBL *in vitro* Effect of EYFZ on proliferation of human PBL was examined by MTT assay[6,7]. Ten ml of peripheral blood was drawn from healthy adult donors into a small glass tube with heparin, and diluted 1:2 with PBS. Cell suspensions were layered on a Ficoll Isopaque (1.077 g/ml, Immuno-Biological Laboratories Ltd.) and centrifuged at 2000 rpm for 30 min. Mononuclear cells were collected from the interphase and washed three times with PBS. The peripheral blood mononuclear cells were suspended in RPMI-1640 medium containing 10% fetal bovine serum (FBS), $NaHCO_3$ (2.0 g/L), HEPES (1.0 g/L), L-glutamine (0.6 g/L), and kanamycin (0.25 mg/L), and cell concentration was adjusted to 1×10^6 cells/ml. These cells were incubated in a 75 cm^2 tissue culture flask (IWAKI Glass Co., Ltd.) for 2 hr at 37℃ in a 5 % CO_2 incubator to allow adherence. Then, non-adherent lymphocytes were gently collected, and cell concentration was adjusted to 5×10^5 cells/ml. 100 μl of cell susperention was put into each well of 96 well flat-bottomed plate (IWAKI) with EYFZ (2000 μg/ml, 400 μg/m, 200 μg/ml), concanavalin A (ConA) (5 μg/ml) (SIGMA CHEMICAL CO.) or culture medium alone, and incubated for 72 hr at 37 ℃ in a 5 % CO_2 incubator. After incubation, the proliferation of PBL was examined by MTT assay: Each well received 10 μl of MTT (SIGMA) solution (5 mg/ml) and incubated for 5 hr at 37 ℃ in a 5 % CO_2 incubator, then 0.1 ml of 0.04 mol/L HCl-isopropanol was added and incubated for 24 hr at 37 ℃ in a 5 % CO_2 incubator and an absorbance at 570 nm was analysed spectrophotometrically.

Reverse transcription-polymerase chain reaction (RT-PCR) for IFN-γ, interleukin-2 (IL-2) and interleukin-4 (IL-4) mRNA expression Human PBL were cultured with either EYFZ (400 μg/ml), ConA (5 μg/ml) or culture medium alone in 25 cm^2 plastic culture flasks (IWAKI) for 72 hr at 37 ℃ in a 5% CO_2 incubator. After incubation, cells (1×10^6 cells) were used for the preparation of RNA. Total RNA was purified using RNeasy Mini Kit (QIAGEN).

The first strand cDNA synthesis was performed by incubating 0.5 μg of RNA in a total reaction volume of 20 μl containing 1 mM dNTP mixture, 1 mM oligo-(dT), 1x reaction buffer for AMV reverse transcriptase, and 4 units (U) of AMV reverse transcriptase XL (Life Science) at 42 ℃ for 4 hr. The sequence of oligo-(dT) was 5'-GCTCTAGATTTTTTTTTTTTTTTTTTTT-3'.

RT-PCR was performed by adding 1U Taq polymerase and 2 μl each of 10 mM oligonucleotide primers as described below. The condition of RT-PCR was as follows: IFN-γ and IL-2, 45 sec at 94 ℃, 45 sec at 60 ℃, 90sec at 72 ℃ for 35 cycles; IL-4, 70 sec at 94 ℃, 70 sec at 60 ℃, 115 sec at 72 ℃ for 35 cycles.

The following primers were used for RT-PCR:
IFN-γ sense,
5'-ATGAAATATACAAGTTATATCTTGGCTTT-3';
antisense, 5'-GATGCTCTTCGACCTCGAAACAGCAT-3'.
IL-2 sense, 5'-ATGTACAGGATACAACTCCTGTCTT-3';
antisense, 5'-GTCAGTGTTGAGATGATGCTTTGAC-3'.
IL-4 sense, 5'-ATGGGTCTCACCTCCCAACTGCT-3';
antisense, 5'-CGAACACTTTGAATTTTCTCTCTCAT-3'.

Analysis of effect of EYFZ on production of IFN-γ, IL-2 and IL-4 by ELISA Human PBL were cultured under the same condition as described above for the analysis of production of IFN-γ, IL-2 and IL-4. After 72 hr incubation, the culture supernatant was collected and stored at −80 ℃ until use. IFN-γ, IL-2 and IL-4 were measured by ELISA according to the manufacturer's instruction (OptEIATM Human IFN-γ, IL-2 and IL-4 Set, PHARMINGEN) to analyse the effect of EYFZ on the cytokine production of human PBL.

Analysis of lymphocyte subsets involved in IFN-γ production Human PBL were prepared in a similar way as described above, and adjusted to a cell concentration of 5×10^5 cells/ml. Then, the PBL were finally re-suspended in RPMI-1640 medium with or without anti-human CD4 (clone H129.19, Pharmingen, Sandiego) (5 μg/ml), anti-human CD8 (clone 53-6.7, Pharmingen, Sandiego) (5 μg/ml) or both anti-human CD4 and anti-human CD8. These monoclonal antibodies were used in an inhibition experiment : For example, the activity of cytotoxic T lymphocytes was shown to be inhibited or blocked by anti-CD8[8]. In parallel with this test, we also examined the effect of addition of EYFZ (400 μg/ml) to those using each monoclonal antibody. In this experiment, the control received the PBL alone or with EYFZ. The cells in 0.2 ml volume per well were incubated using 96 well flat-bottomed plate (IWAKI) for 72 hr at 37 ℃ in a 5 % CO_2 incubator. Following incubation, the culture supernatant was collected and stored at −80 ℃ until use. Then, IFN-γ was similarly measured by ELISA method as described before to know the effect of monoclonal antibodies on the IFN-γ production with or without EYFZ.

Statistical analysis The data were statistically analysed based on the Student's t test, and differences were

recognized significant with p value less than 0.05. Here, results were expressed as mean ± standard deviation (SD).

Results

Effects of EYFZ on proliferation of human PBL *in vitro* Proliferation of human PBL was examined by MTT assay. Peripheral blood was drawn from healthy donors, and separated PBL were cultured with either EYFZ (2000 μg/ml, 400 μg/ml, 200 μg/ml), ConA (5 μg/ml) or culture medium alone in a 96 well flat-bottomed plate. After 72 hr-incubation, the proliferation of PBL was measured by MTT assay. All samples were measured in triplicate and the experiment was performed 3 times. As shown in Fig. 1, EYFZ directly stimulated PBL to proliferate without any mitogen, and proliferative effect was almost the same as that of ConA. ($P<0.01$ versus the culture medium alone control)

Effect of EYFZ on expression of IFN-γ, IL-2 and IL-4 mRNA Expression of IFN-γ, IL-2 and IL-4 mRNA of PBL was examined by RT-PCR. As shown in Fig. 2, after 72 hr-incubation, EYFZ (400 μg/ml) induced expression of IFN-γ mRNA as compared with culture medium alone, but not expression of IL-2 and IL-4. ConA (5 μg/ml), as a control, induced significant expression of IFN-γ, IL-2 and IL-4 mRNA more than in EYFZ addition or in culture medium alone.

Effect of EYFZ on production of IFN-γ, IL-2 and IL-4 in a culture supernatant It was examined whether or not EYFZ would affect the IFN-γ, IL-2 and IL-4 production by PBL. As shown in Fig.3, after 72 hr-incubation, EYFZ (400 μg/ml) promoted significantly the production of INF-γ in culture supernatant of PBL as compared with that in the culture supernatant without EYFZ or ConA ($P<0.01$), but did not promote IL-2 or IL-4 production. ConA significantly promoted the production of IFN-γ, IL-2 and IL-4 higher than in EYFZ addition or in culture medium addition alone.

Effect of anti-CD4 and anti-CD8 on IFN-γ production with or without EYFZ This experiment was performed to identify the lymphocyte subsets associated with INF-γ production by EYFZ. As shown in Fig. 4, though INF-γ was not found after 72hr culture in the absence of EYFZ, the addition of EYFZ promoted the INF-γ production. However, the production of INF-γ was inhibited by anti-CD4 or anti-CD8 monoclonal antibodies as compared to the addition of EYFZ without antibody. The presence

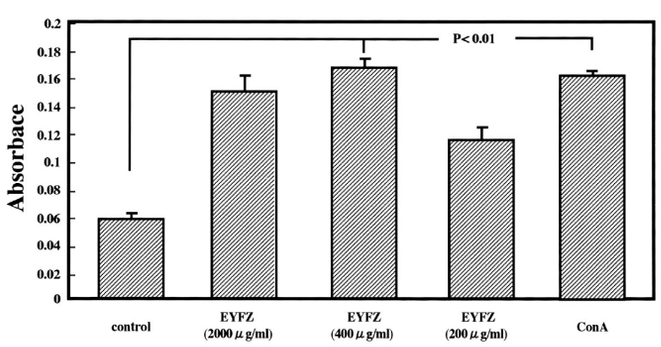


Fig. 1. Effect of Ekki-Youketsu-Fusei-Zai (EYFZ) on proliferation of human peripheral blood lymphocytes (PBL) *in vitro*. Human PBL (5×10^5 cells/ml) were cultured with EYFZ (2000 μg/ml, 400 μg/ml, 200 μg/ml), ConA (5 μg/ml) or culture medium alone in 96 well flat-bottomed plate. After 72 hr incubation, the proliferation of lymphocytes was measured by MTT assay. Each column and vertical bar represents the mean ± standard error of triplicates. ($P<0.01$ by Student's two tailed test) (control: culture medium without EYFZ or ConA)

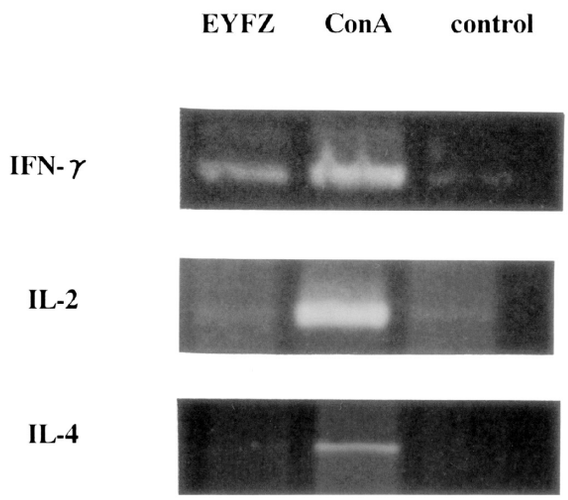
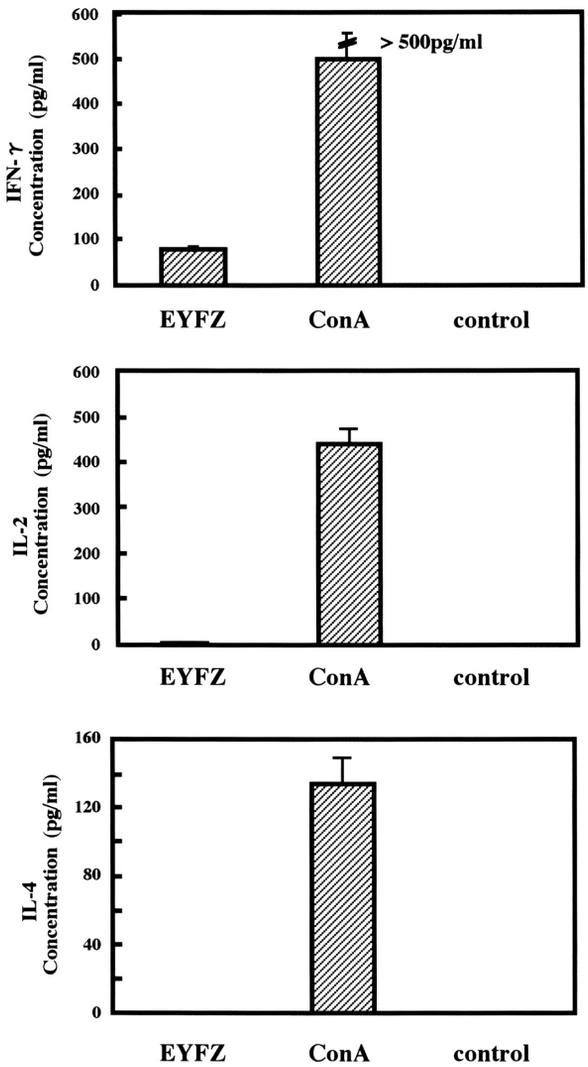


Fig. 2. Effect of Ekki-Youketsu-Fusei-Zai (EYFZ) on expression of IFN-γ, IL-2 and IL-4 mRNA Human PBL (5×10^5 cells/ml) were cultured with EYFZ (400 μg/ml), ConA (5 μg/ml) or culture medium alone in 25 cm^2 culture flask. After 72 hr incubation, RT-PCR analysis was performed to determine the expression of IFN-γ, IL-2 and IL-4 mRNA.



of both antibodies also strongly inhibited the INF-γ production, although this inhibition was not perfect.

Discussion

In the present study, we examined the effect of EYFZ on the proliferation and cytokine production of the human PBL. Results clearly showed that culturing with EYFZ exerted a statistically significant proliferative effect on PBL. This proliferative response of PBL was comparable to that of ConA. ConA has been used as the specific mitogen for T lymphocytes[9,10]. In our experiment, culturing with EYFZ plus ConA gave similar proliferative effect on PBL as EYFZ alone and ConA alone (data not shown). This suggests that both stimulated T lymphocytes and their effects were maximum.

It has been reported that all the stimuli that induce proliferation of T cells initiate the synthesis of IL-2 and the formation of IL-2R on the cell surface[11]. In

Fig. 3. Effect of Ekki-Youketsu-Fusei-Zai (EYFZ) on production of IFN-γ, IL-2 and IL-4 in culture supernatant. Human PBL were cultured the same conditions as RT-PCR for IFN-γ, IL-2 and IL-4 mRNA expression. After 72hr incubation, ELISA analysis was used to determine production of IFN-γ, IL-2 and IL-4. Values are mean ± standard error of triplicates and represent one of three indepent experiments. ($P<0.01$ by Student's two-tailed test)

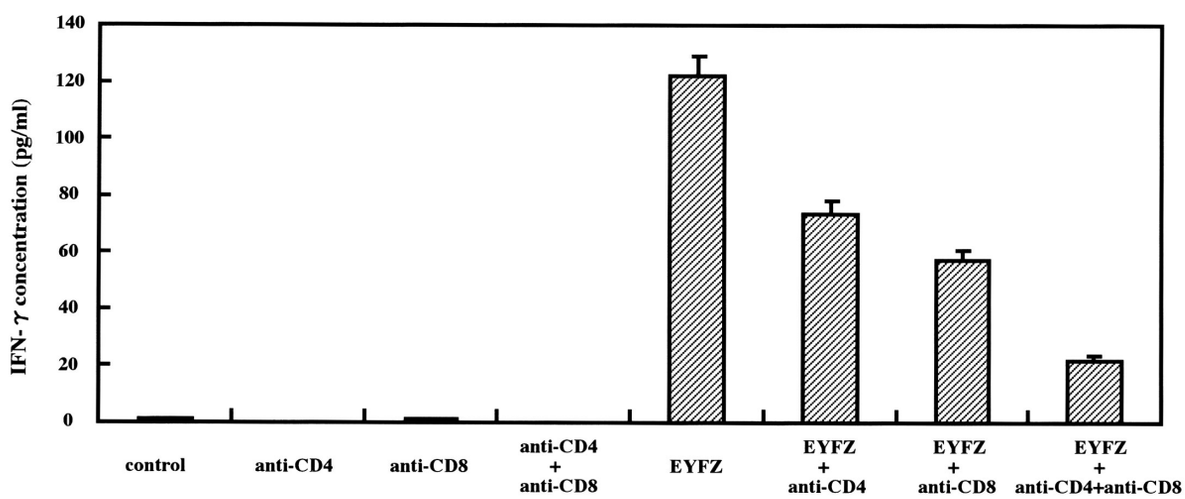


Fig. 4. Effect of anti-CD4 and anti-CD8 on IFN-γ production with or without EYFZ. The human PBL (5×10^5 cells/ml) were suspended in RPMI-1640 medium with or without mouse monoclonal anti-human CD4, anti-human CD8, or both anti-human CD4 and anti-human CD8. 0.2 ml of cell suspension was put into each well of 96 well flat-bottomed plate with or without EYFZ (400 μg/ml), and incubated for 72 hr at 37 ℃ in a 5 % CO_2 incubator. After 72 hr incubation, ELISA analysis was used to determine production of IFN-γ. Each column and vertical bar represents the mean ± standard error of triplicates.

our experiment, RT-PCR and ELISA revealed that ConA induced the expression of IL-2 mRNA and the production of IL-2 in a culture medium, but EYFZ did not induce the expression or the production of IL-2. About reasons, we don't know exactly now. Because the EYFZ is a mixture of six kinds of crude drugs and an effective component of each crude drug was not clearly demonstrated to induce cytokine production. However, some studies on some murine T cell clones and human PBL shows the stimulation of cell proliferation with monoclonal antibody[12,13]. Therefore, since the proliferative effect of EYFZ on human PBL is possibly due to the IL-2-indepentent pathway. We will study this possibility in the future.

In addition, we also examined the effects of EYFZ on both the expression of IFN-γ, IL-2 and IL-4 mRNA and the production of IFN-γ, IL-2 and IL-4 in culture supernatant. Results showed that EYFZ induced only the expression of IFN-γ mRNA and production of IFN-γ in culture supernatant. The expressions and productions of IL-2 or IL-4 were not observed in our experiment. As has already been demonstrated, helper T cells are classified into two subsets (Th1 and Th2) on the basis of the cytokines synthesized by T cells. Namely, Th1 cells secreting IL-2 and/or INF-γ causes the cellular immunity, whereas Th2 cells secreting IL-4, IL-5, IL-6 and/or IL-10 participate in the humoral immunity[14]. As our investigation using anti-CD4 and anti-CD8 clearly showed that $CD4^+$ and $CD8^+$ T cell subsets were at least associated with the IFN-γ production in the presence of EYFZ. There is one possibility that EYFZ contains some effective components to induce only IFN-γ from those T cells. The mechanism of only IFN-γ production by T cells with EYFZ will be needed to be elucidated in the future.

In conclusion, results of our studies suggest that EYFZ can stimulate the human PBL to proliferate, and induce the expression and production of IFN-γ by stimulating T cells subsets of $CD4^+$ and $CD8^+$ T cells. The effects of EYFZ on T cells of patients *in vivo* and *in vitro* need to be further pursued.

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中国の伝統的漢方薬「益気養血扶正剤」がヒト末梢血中のリンパ球機能に及ぼす影響

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中国の伝統的漢方薬である「益気養血扶正剤」（オウギ，ジュクジオウ，トウキ，ジュクシャ，タイソウ，ロクジョウの6種類から構成される：以下EYFZと略す）が抗腫瘍活性を示すことを，担癌マウスの延命効果，NK活性促進並びにマクロファージ細胞株J774.1細胞機能活性化から明らかにしたので，本研究では特にヒトの末梢血中のリンパ球機能に着目し，*In vitro* でEYFZがリンパ球機能にいかなる影響を及ぼすかについて追究した．最初に，分離した末梢血中のリンパ球増殖に対する影響をMTTアッセイで調べた結果，EYFZはマイトーゲンが存在していない条件下でも著しくリンパ球の増殖を引き起こすことがわかった．さらに，リンパ球由来サイトカインであるインターロイキン−2（IL-2），IL-4及びインターフェロン-γ（IFN-γ）の産生に対するEYFZの影響をRT-PCR法並びにELISA法によって検討した．その結果，EYFZは特にINF−γmRNAのみの誘導とIFN-γのみの産生を引き起こす効果のあることが判明した．このINF−γがいかなる種類のリンパ球由来したものかを同定するために，抗CD4抗体と抗CD8抗体を用いて阻害実験を行った結果，これらの抗体によってINF−γの産生は阻害されることがわかった．以上の結果から，EYFZにはヒト末梢血中の$CD4^+$あるいは$CD8^+$のTリンパ球を芽球化すると同時に，INF−γの産生を促進してTh1優位を引き起こす結果細胞性免疫を高める効果のあることが強く示唆された．

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