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

Hong Deng, Kaori Nakajima, Xinling Ma, Kenichiro Hasumi, Toshitaka Akatsuka, and Haruhisa Wago

**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 expression 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)

*J Saitama Med School 2001;28:125-130*
(Received April 24, 2001)

**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. 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. 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.

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. 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
Effect of EYFZ on proliferation of human PBL in vitro

Effect of EYFZ on proliferation of human PBL was examined by MTT assay. 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 Ficol 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), 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°C 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 suspension was put into each well of 96 well flat-bottomed plate (IWAKI) with EYFZ (2000 μg/ml, 400 μg/m, 200 μg/m), concanavalin A (ConA) (5 μg/ml) (SIGMA CHEMICAL CO.) or culture medium alone, and incubated for 72 hr at 37°C 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°C 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°C 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°C 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°C 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°C, 45 sec at 60°C, 90 sec at 72°C for 35 cycles; IL-4, 70 sec at 94°C, 70 sec at 60°C, 115 sec at 72°C for 35 cycles.

The following primers were used for RT-PCR:
- IFN-γ sense, 5'-ATGAAATATACAAATATCTGGTGCAT-3';
- IFN-γ antisense, 5'-GATGCTCTTTCGACCTCGAAACGATAC-3';
- IL-2 sense, 5'-ATGACAGTACAACCTCGATTCTTT-3';
- IL-2 antisense, 5'-GTCAGTGTGGATGATGATGACT-3';
- IL-4 sense, 5'-ATGGGTCTCACCTCCCAACTGCT-3';
- IL-4 antisense, 5'-GATGCTCTTTCGACCTCGAAACGATAC-3';
- 5'-CGAAGACTTTGATTTTCTCTCAT-3'.

RT-PCR was similarly measured by ELISA method (OPTIATM 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$. In parallel with this test, we also examined the effect of addition of EYFZ (400 μg/ml) to those using each monoclonal antibody. In this experiment, the control received the PBL alone or with EYFZ. The cells in 0.2 ml volume per well were incubated using 96 well flat-bottomed plate (IWAKI) for 72 hr at 37°C in a 5% CO$_2$ incubator. Following incubation, the culture supernatant was collected and stored at −80°C until use. Then, IFN-γ and IL-2 were measured by ELISA according to the manufacturer’s instruction (OptiATM Human IFN-γ, IL-2 and IL-4 Set, PHARMINGEN) to analyse the effect of EYFZ on the cytokine production of human PBL.

Statistical analysis

The data were statistically analysed based on the Student’s t test, and differences were 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
of both antibodies also strongly inhibited the INF-\(\gamma\) 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-\(\gamma\), IL-2 and IL-4 in culture supernatant. Human PBL were cultured the same conditions as RT-PCR for IFN-\(\gamma\), IL-2 and IL-4 mRNA expression. After 72hr incubation, ELISA analysis was used to determine production of IFN-\(\gamma\), 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-\(\gamma\) 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 \(\mu\)g/ml), and incubated for 72 hr at 37 °C in a 5 % CO\(_2\) incubator. After 72 hr incubation, ELISA analysis was used to determine production of IFN-\(\gamma\). 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\textsuperscript{12,13}. Therefore, since the proliferative effect of EYFZ on human PBL is possibly due to the IL-2-independent pathway. We will study this possibility in the future.

In addition, we also examined the effects of EYFZ on both the expression of IFN-\(\gamma\), IL-2 and IL-4 mRNA and the production of IFN-\(\gamma\), IL-2 and IL-4 in culture supernatant. Results showed that EYFZ induced only the expression of IFN-\(\gamma\) mRNA and production of IFN-\(\gamma\) 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-\(\gamma\) causes the cellular immunity, whereas Th2 cells secreting IL-4, IL-5, IL-6 and/or IL-10 participate in the humoral immunity\textsuperscript{10}. 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-\(\gamma\) production in the presence of EYFZ. There is one possibility that EYFZ contains some effective components to induce only IFN-\(\gamma\) from those T cells. The mechanism of only IFN-\(\gamma\) 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-\(\gamma\) by stimulating T cell subsets of CD4\(^+\) and CD8\(^+\) T cells. The effects of EYFZ on T cells of patients \textit{in vivo} and \textit{in vitro} need to be further pursued.

References


