Regulatory Effect of Fu-Ling on Th1 and Th2-type Cytokine Induced Immune Response

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ABSTRACT

We examined possible effect of Fu-Ling a widely used Chinese herbal medicine on Th1-type and Th2-type cytokine production by murine spleen cells. Mice were injected with a 50% ethanol extract of Fu-Ling for three consecutive days. The serum was collected and spleen cells were isolated and cultured in the presence of concanavalin A. The serum levels of interleukine-2 (Th1-type cytokine) and interleukine-4, interleukine-10 (Th2-type cytokine) were elevated significantly (P<0.05) after treatment. The production of IL-2, interferon-γ, IFN-γ and IL-4, IL-10 by spleen cells, as measured by ELISA and RT-PCR, was significantly augmented by the Fu-Ling ethanol extract. Fu-Ling extract significantly (P<0.01) augmented secretion of immunoglobulin G1, immunoglobulin G2b and immunoglobulin G2a by in vitro spleen cells. Fu-Ling may regulate antibody production by the induction of Th1-type (IL-2, IFN-γ) and Th2-type (IL-4, IL-10) cytokine production.

Keywords: Fu-Ling, immunoglobulin, cytokines

Introduction

Chinese herbal medicines have been claimed beneficial to the immune and gastrointestinal systems in chronic disease patients (Liou and Tseng, 2002). Although clinical trials have been performed in Asian countries for hundreds of years, detailed pharmacological studies of traditional tonic recipes are still in the early stages. Si-Jun-Zi-Tang is one of the most widely used tonic recipes in Chinese herbal medicine. Si-Jun-Zi-Tang consists of four major herbs: Ginseng (Panax ginseng C. A. Mey), Fu-Ling (Poria cocos (Schw.) Wolf), Bai-Zhu (Atractylodes macrocephala Koidzumi) and Gan-Cao (Glycyrrhiza uralensis Fischer). Under in vitro conditions, we demonstrated that Si-Jun-Zi-Tang regulates immunoglobulin A (IgA) production in human peripheral blood mononuclear cells (Lu et al., 1994). In addition, Si-Jun-Zi-Tang enhances granulocyte macrophage colony-stimulating factor (GM-CSF) secretion by human peripheral blood mononuclear cells (Tseng and Li, 1996).

Of the components in Si-Jun-Zi-Tang, Fu-Ling is the active ingredient capable of regulating the immune response (Lu et al., 1994). Fu-Ling is widely used in Chinese herbal drugs. It is produced from the seledera of Poria cocos (Schw.), a fungus that grows on the roots of pine trees (Liou and Tseng, 2002). Based on its major pharmacological effects, Fu-Ling has been classified as a sedative and diuretic. In addition, Fu-Ling is a major ingredient for various tonic recipes. Fu-Ling significantly enhances the secretion of interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), but suppressed the secretion of transforming growth factor-β (TGF-β) in vitro (Yu and Tseng, 1996). We have also demonstrated that Fu-Ling significantly induces an increase in immunoglobulin G (IgG) and immunoglobulin A (IgA) secretion by spleen cells but does not affect immunoglobulin M (IgM) secretion (Liou and Tseng, 2002). Therefore, we suggest that Fu-Ling may affect the function of B-lymphocytes by stimulating cytokine production.

This study focused on the effect of Fu-Ling on cytokine production. The T helper 1-type (Th1-type) cytokine is responsible for
cell-mediated immunological responses (Stephanie and Kim, 1997). This subset of helper cells is also involved in inflammatory reactions, macrophage activation, delayed-type hypersensitivity and cytotoxic T cell activation. The cytokines secreted by Th1 cells also boost production of immunoglobulin G2a (IgG2a) antibody production in mice (Koh et al., 2001). The Th1 subset produces interleukine-2 (IL-2), interferon-γ (IFN-γ), TNF-α, GM-CSF and interleukine-3 (IL-3) (Lanzavecchia and Sallusto, 2000). The T helper 2-type (Th2-type) cytokine-mediated pathway is essentially a humoral pathway. The Th2-type cytokines promote B cell growth and production of immunoglobulin G1 (IgG1) and immunoglobulin G2b (IgG2b), IgA and immunoglobulin E (IgE) in mouse model (Laurat et al., 2001). This subset produces IL-3, IL-4, IL-5, IL-10, and interleukine-13 (IL-13). (Stephanie et al., 1997)

In this study, we investigated the relatively short-term effect of Fu-Ling by peritoneally injecting pathogen-free mice for three consecutive days with a 50% ethanol extract of Fu-Ling. Both the Th1-type (IL-2, IFN-γ) and Th2-type (IL-4, IL-10) cytokine production by the spleen cells were measured after treatment. The possible relationship between cytokine production and immunoglobulin production by Fu-Ling-treated mice was discussed.

Materials and Methods

Preparation of Fu-Ling Extract

Fu-Ling was purchased from the Lao-Chen-Ge Chinese herbal drugstore, De-Hua Street, Taipei, and ground into a dried powder. This powder (5 g) was mixed and suspended in 100 ml of 50 % ethanol. The drug suspension was boiled until half the volume of liquid remained. The suspension was then spun at 10,000 g for 30 min and the supernatant was collected and dried with a Speed Vac. Dried extracts were reconstituted using a sterile phosphate buffer saline (PBS) to make a stock of 100 mg drug/ml. This was sterilized with a 0.2 µm Millipore filter before being used.

Animal Treatment and Cell Culture

Male BALB/cByJ mice with 8 to 12 weeks old were purchased from the National Laboratory Animal Center, Taipei. The mice were divided into four groups. Three were injected peritoneally with 1 ml of Fu-Ling extract, ranging from 0.1g, 0.5g to 1 g/kg body weight daily (g/kg/day) for three days. The control group was injected with an equal volume of PBS. Mice were sacrificed at day four. Serum from each mouse was collected and the spleen cells were isolated. The spleen cells (5x10⁵ cells/ml) were cultured in a medium containing RPMI-1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, antibiotics and lipopolysaccharide (LPS; 1 µg/10⁵ cells) for five days. The IgG subtype (IgG1, IgG2a and IgG2b) concentrations in the culture supernatants were estimated using the enzyme-linked immunosorbent assay (ELISA) technique (Liou and Tseng, 2002).

To investigate the effects of Fu-Ling on cytokines production, mice were treated as described here, but the spleen cells were cultured in the presence of 1 µg/ml concanavalin A (Con A) for three days. Culture supernatants of the spleen cell were collected, and the cytokine concentrations were measured using the ELISA technique.

Enzyme-Linked Immunosorbent Assay (ELISA)

The IgG1, IgG2a and IgG2b concentrations were measured using a sandwich ELISA technique. The capturing antibody for assay was a rabbit anti-mouse IgG + IgA + IgM antibody. The secondary antibody for assay was the horseradish peroxidase-conjugated goat anti-mouse IgG1, anti-mouse IgG2a and anti-mouse IgG2b. Briefly, a 96-well microtiter plate was precoated with a 100 ng/well of capture antibodies at 4°C overnight. The plate was washed with PBS-0.05% Tween 20 solution and blocked with PBS-1% gelatin. After blocking, properly diluted samples and standards were added (100µl/well). Standards for IgG1, IgG2a or IgG2b a ranged from 0.2µg/ml to 0.03125 µg/ml. The plate was then incubated at 37°C for 2 h. At the end of incubation, horseradish peroxidase-conjugated secondary antibody was added (100 µl/well). After 1 h of incubation at 37°C, the color was developed using a substrate solution containing 0.1 M citrate buffer, pH 4.5, 0.03% hydrogen peroxide and 0.1% of o-phenylenediamine. The absorbance at 490nm in each well was read using an ELISA reader. Data were analyzed using log-logit model.

For quantitative analysis of IL-2, IFN-γ, IL-4
and IL-10, we used the cytokine ELISA set purchased from R&D Systems (MN, USA). The capture antibody was a rat monoclonal antibody to mouse cytokine. The detection antibody was a biotinylated goat anti-cytokine polyclonal antibody. The color was developed by incubating the plate with a horseradish peroxidase-conjugated streptavidin, followed by incubating a substrate solution containing hydrogen peroxide and tetramethylbenzidine. The reaction continued for 30 min at room temperature, and was stopped by adding 100 μl of 2N of sulfuric acid. The absorbance at 450nm in each well was read using an ELISA reader. Data was analyzed using log-logit model.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)
Synthesis of cDNA
The level of Th1-type (IL-2, IFN-γ) and Th2-type (IL-4, IL-10) mRNA in the spleen cells was estimated using RT-PCR analysis 36 h after culture in the presence of 1μg/ml of Con A. Spleen cells (1 x 10⁷ cells) isolated from Fu-Ling-treated animals were washed twice with 1 x RNase-free PBS. The cell pellet was mixed with 1 ml of TRIzol reagent, and the mixture was forced to pass through a 25 G needle 5 times to release RNA from the cells. This homogenate was vigorously mixed with 0.2 ml chloroform. After sitting at room temperature for 10 min, the mixture was spun at 4°C for 15 min to separate the organic from the aqueous layers. The aqueous layers were removed into a new tube and RNA was precipitated with 0.5 ml of isopropanol. The precipitate was then resuspended in 30 μl of RNase-free water, and a 5 μl aliquot was removed for RNA quantification using GeneQuant II. RNA in RNase-free water (2 μg in 10 μl) was mixed with 2.5 μl of oligo (dT)₁₅ solution. The solution was heated at 70°C for 10 min, followed by cooling at room temperature for 10 min and then transferred onto ice. A reaction mixture containing 4 dNTPs, DTT, reverse transcriptase and RNasin was subsequently mixed with the RNA. The reaction was carried out at 37°C for 60 min to synthesize the cDNA.

Polymerase Chain Reaction (PCR)
The cDNA (5 μl) was mixed with 0.5 μl 4 dNTP, 10 μl primer mix (2.5 μM each), 0.5 μl Tag polymerase, 2 μl MgCl₂ (2 mM), and 5 μl PCR buffer. The DEPC-treated water was then added to make a volume up to 50 μl in total. The primers used for PCR amplification were: IL-10 sense primer, 5'-ATGCAGGCATTAAAGGTACTTG-3'; IL-10 antisense primer, 5'-TAGACACCTTGTTGGAGCTTA-3'; IL-4 sense primer, 5'-TGCCCTCAAGAAACACAAGTG-3'; IL-4 antisense primer, 5'-TTCTGACTCTGGTGCTCCTAG-3'; IL-2 sense primer, 5'-GAACATCTGCTGCTTGCCTCA-3'; IL-2 antisense primer, 5'-TCAATTCTTGGCTGCTTGG-3'; IFN-γ sense primer, 5'-GCAGGCAAAATTGCTCCTAC-3'; IFN-γ antisense primer, 5'-ATGCCTCTCGACCTCTCGA AAC-3'; β₂ microglobulin (internal control) sense primer, 5'-TGACCGCGCTTATGTCTATTCTC-3'; and β₂ microglobulin antisense primer, 5'-CACAGTTGACCCAGGATAG-3'. The PCR conditions were denaturation at 94°C for 30 sec, annealing at 60°C for 45 sec and primer extension at 72°C for 45 sec. After 35 cycles (40 cycles for IL-10) of amplification, the PCR products were subjected to gel electrophoresis through 1.5% agarose containing ethidium bromide at 80 V. The ampiclons were visualized under UV light.

Statistics Analysis
Data from the control and drug-treatment groups were tested by ANOVA. The difference between the two groups was assessed using the Student's t-test. Probability values of < 0.05 were considered to be significant.

Results
Fu-Ling induced an increase in cytokine production
Fu-Ling induced an increase in immunoglobulin production in both sera and spleen cells isolated from the Fu-Ling-treated mice and assayed for cytokine level and cytokine secretion, respectively. The serum level of IL-2 (Th1-type cytokines) were significantly elevated after the mice were peritoneally injected with ≥ 0.5 g/kg/day dose of 50% ethanol-extracted Fu-ling for three consecutive days. The serum concentration of IL-2 increased 10 to 20 fold. However, the effect of Fu-Ling on IFN-γ secretion was not significant (Figure 1). The serum levels of IL-4

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Figure 1. The level of Th1-type cytokines in sera collected from Fu-Ling treated mice. The BALB/c mice were peritoneally injected for three consecutive days with Fu-Ling ethanol extract in doses ranging from 0.1g/kg/day to 1g/kg/day. The control group consisted of mice injected with an equal volume of phosphate buffer saline (PBS). The sera were collected and assayed for IL-2 and IFN-γ concentrations using enzyme-linked immunosorbent assay (ELISA). Data were mean ± standard error mean (SEM) of six replicates. * indicates $P<0.05$ from the control group.

Figure 2. The level of Th2-type cytokines in sera collected from Fu-Ling treated mice. The BALB/c mice were peritoneally injected for three consecutive days with Fu-Ling ethanol extract in doses ranging from 0.1g/kg/day to 1g/kg/day. The control group consisted of mice injected with an equal volume of PBS. The sera were collected and assayed for IL-4 and IL-10 concentrations using ELISA. Data were SEM of six replicates. * indicates $P<0.05$ from the control group.

Figure 3. Fu-Ling induced an increase in Th1-type cytokine secretion by spleen cells in vitro. The BALB/c mice were peritoneally injected for three consecutive days with Fu-Ling ethanol extract in doses ranging from 0.1g/kg/day to 1g/kg/day. The control group consisted of mice injected with an equal volume of PBS. Spleen cells were isolated and in vitro cultured for 72 h in the presence of 1μg/ml of concanavalin A (Con A). Supernatants were harvested and assayed for Th1 type cytokine (IL-2 and IFN-γ) by ELISA. Data were mean ± SEM of six replicates. * indicates $P<0.05$ from the control group.

Figure 4. Fu-Ling induced an increase in Th2-type cytokine secretion by spleen cells in vitro. The BALB/c mice were peritoneally injected for three consecutive days with Fu-Ling ethanol extract in doses ranging from 0.1g/kg/day to 1g/kg/day. The control group consisted of mice injected with an equal volume of PBS. Spleen cells were isolated and in vitro cultured for 72 h in the presence of 1μg/ml of Con A. Supernatants were harvested and assayed for Th2-type cytokine (IL-4 and IL-10) by ELISA. Data were mean ± SEM of six replicates. * indicates $P<0.05$ from the control group.
and IL-10 (Th2-type cytokines) were also elevated by the Fu-Ling treatment. Mice injected with ≥ 0.5 g/kg/day of Fu-Ling extract showed a significant increase in serum levels of IL-10 and IL-4. However, the serum level of IL-4, in a range between 0.1 to 3 pg/ml, was approximately 100-fold lower than that of IL-10 (Figure 2). For spleen cells, the Th1 type and Th2 type cytokine secretion by spleen cells was significantly augmented after the Fu-Ling treatment of doses as low as 0.1 g/kg/day (Figure 3 and 4).

**Fu-Ling augmented expression of Th1 and Th2 type cytokine mRNA**

Results of RT-PCR correlated well with ELISA, expression of IL-2 and IFN-γ mRNA increased Fu-Ling treatment dose. The levels of IL-4 and IL-10 mRNA expression were elevated in the spleen cells from Fu-Ling-treated mice as compared to that of the control group (Figure 5).

**Fu-Ling extract increased secretion of IgG subtypes by spleen cells**

After *in vivo*, the concentrations of IgG1 and IgG2b in culture supernatants of spleen cells increased with dose of Fu-Ling extract (Figure 6 and 7). Therefore, IL-4 production was enhanced by the Fu-Ling extract. The IFN-γ was a potential inducer of class-switching from IgM to IgG2a. The amount of IgG2a secretion also significantly increased after the mice were treated with Fu-Ling extract, confirming that Fu-Ling treatment did enhance IFN-γ production (Figure 8).

**Figure 5.** Detection of cytokine mRNA expression in spleen cells isolated from Fu-Ling mice. The BALB/c mice were peritoneally injected for three consecutive days with doses of Fu-Ling ethanol extract in 100 μl/kg/day. The control group consisted of mice injected with an equal volume of PBS. Spleen cells were isolated and cultured *in vitro* for 36 h in the presence of 1 μg/ml of Con A. The total mRNA was isolated from the spleen cells (1 x 10⁶ cells). The cDNA was synthesized and then amplified using polymerase chain reaction. The level of β₂m mRNA was the internal control. Lane 1: control; Lane 2: 0.1 g/kg/day; Lane 3: 0.5 g/kg/day; Lane 4: 1 g/kg/day; interleukine (IL-2); interleukine-4 (IL-4); interleukine-10 (IL-10); interferon-γ (IFN-γ); β₂m microglobulin (β₂m).

**Figure 6.** Fu-Ling induced an increase in IgG1 secretion by spleen cells *in vitro*. The BALB/c mice were peritoneally injected for three consecutive days with Fu-Ling ethanol extract in doses ranging from 0.1 g/kg/day to 1.0 g/kg/day. The control group consisted of mice injected with an equal volume of PBS. Spleen cells were isolated after the treatment and then incubated for 5 days with 1 μg/ml of lipopolysaccharide. Supernatants were harvested and assayed for IgG1 concentrations using ELISA. Data were mean ± SEM of six replicates. * indicates P<0.01 from the control group.
Figure 7. Fu-Ling induced an increase in IgG2b secretion by spleen cells in vitro. The BALB/c mice were peritoneally injected for three consecutive days with Fu-Ling ethanol extract in doses ranging from 0.1g/kg/day to 1g/kg/day. The control group consisted of mice injected with an equal volume of PBS. Spleen cells were isolated after the treatment and then incubated for 5 days with 1 µg/ml of lipopolysaccharide. Supernatants were harvested and assayed for IgG2b, concentrations using ELISA. Data were mean ± SEM of six replicates. * indicates P<0.01 from the control group.

Figure 8. Fu-Ling induced an increase in IgG2a secretion by spleen cells in vitro. The BALB/c mice were peritoneally injected for three consecutive days with Fu-Ling ethanol extract in doses ranging from 0.1g/kg/day to 1g/kg/day. The control group consisted of mice injected with an equal volume of PBS. Spleen cells were isolated after the treatment and then incubated for 5 days with 1 µg/ml of lipopolysaccharide. Supernatants were harvested and assayed for IgG2a, concentrations using ELISA. Data were mean ± SEM of six replicates. * indicates P<0.01 from the control group.

Discussion

Fu-Ling has been used in eastern Asian countries for thousands of years, however, both the biological and pharmacological effects of Fu-Ling and its active ingredients need to be studied in detail. Fu-Ling is a potent immunoregulatory drug (Tseng and Li, 1996). We demonstrated peritoneally that Fu-Ling significantly induced an increase in IgG and IgA secretion, but showed no effect on IgM secretion by murine spleen cells (Liou and Tseng, 2002). Here, we suggest that Fu-Ling regulated antibody production by augmenting Th1 type (IL-2, IFN-γ) and Th2 type (IL-4, IL-10) cytokine production.

Fu-Ling obviously elevated the level of cytokine mRNA by either stimulating cytokine gene expression or stabilizing mRNA. In turn, the increase in the level of mRNA. enhanced the production and secretion of cytokine, subsequently elevating the serum levels of cytokine. It is well documented that IFN-γ induces class-switching from IgM producer to IgG1 and IgG2b producers. Therefore, Our results supported the hypothesis that Fu-Ling induces production of IFN-γ and IL-4, which in turns promotes B-cell class-switching and differentiation.

The IL-2 is the cytokine mainly produced by Th1 cells. However, it has been demonstrated that IL-2 also plays an essential role in B-cell differentiation (Stephanie and Kim, 1997). The IL-2 showed a synergistic effect with IL-4 on B-cell class-switching (Itoh and Hirohata, 1995). Moore et al.(2001) indicated that IL-10 in association with CD40L, strongly induced B-cell differentiation. They also demonstrated that IL-2 and IL-4 enhanced the regulatory effects of IL-10 on B-cell functions. Therefore, induction of IL-2 and IL-10 production by Fu-Ling should reinforce B-cell differentiation and augment antibody production.

The in vivo study is the protocol that directly investigates the biological effect of a drug on the entire immune system. However, the method of administration may play the crucial role in the pharmacological effects of Chinese medicine. In
In this study, Fu-Ling extract was peritoneally injected into experimental animals. However, the general route of taking Fu-Ling, however, is by oral administration. Therefore, our study has shown to be oral feeding. The preliminary data of oral administration indicated that Fu-Ling extract augmented IFN-γ production but did not affect IL-10 (in preparation). The differences between peritoneal and oral administration suggest that certain ingredients in Fu-Ling ethanol extract were not absorbed by the gastrointestinal system. The ingredients of Fu-Ling are currently being purified, and undergoing intensive study.

References

茯苓對Th1及Th2型細胞激素誘導免疫反應之調節作用

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摘 要

茯苓為廣範使用的中藥材之一，本實驗室先前研究証明茯苓可顯著促進小鼠脾臟細胞IgG和IgA的分泌。此外，茯苓亦可顯著促進人類周邊血液單核球分泌IL-1γ、TNF-γ和IL-6，但對TGF-β的分泌影響不顯著。本研究目的在透過一系列實驗，証明茯苓可調節小鼠脾臟細胞分泌Th1型和Th2型細胞激素之能力。老鼠腹腔注射50%酒精萃取之茯苓萃取液，連續三天。分離脾臟及收集血清，經過ConA刺激培養。結果顯示，茯苓顯著提升血清中IL-2（Th1型細胞激素）及IL-4、IL-10（Th2型細胞激素）的濃度。藉由ELISA和RT-PCR技術進一步確認，結果証明茯苓同時促進小鼠脾臟細胞Th1型（IL-2、IFN-γ）和Th2型（IL-4、IL-10）之分泌量及mRNA表現量。延伸細胞激素變化調節IgG亞型分泌量的影響，發現茯苓萃取液顯著促進脾臟細胞分泌IgG1、IgG2a及IgG2b，因此推論茯苓萃取液會藉由Th1型和Th2型細胞激素的分泌調節抗體的製造。

關鍵詞：茯苓，免疫球蛋白，細胞激素

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