The Effect of Bojungykgitang-Chunbang on Activity of CD4+ T cell

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BJYGC is often clinically used as a treatment of allergic rhinitis. This study was aimed to find out the effect BJYGC would have on the helper T cell, and how it can promote the subsets of helper T cells to regain their balance that they lost due to immunological diseases. Splenocytes were prepared from BALB/c mice was cultured without stimulation in the presence of BJYGC for 48 hr. The viability of CD4 T cells from Balb/c mouse were measured at various concentrations of BJYGC using the MTS assay. It was somewhat increased up to concentration of 400 ㎍/ml, but did not show any significant difference. Proliferation was measured using the MTS assay, CD4 Th cells were stimulated with anti-CD3/28 in the presence of BJYGC for 48 hr. As evidence for rapid T cell activation, CD25 expression by flow cytometry was evaluated at 10, 50, 100 and 200 ㎍/㎖ of BJYGC. Th cell differentiation experiments were performed to examine whether BJYGC can affect the Th polarization process. CD4 T cells were activated in culture under neutral, Th1-polarized or Th2-polarized conditions in the presence of BJYGC at 10, 100 and 200 ㎍/㎖. Cytokine production was measured by ELISA. This experiment proved that BJYGC could inhibit the secretion of both IL-4 and IFN-γ in neutral condition and polarized condition, too. Considering that BJYGC shows an excellent effect on treating allergies, the author can conclude that its pharmacological action may be associated with decreased IL-4 and, it may also regulate IFN-γ depending the host's need. Also, it was discovered that Th1 cell was pathologic in chronic inflammatory tissue specific diseases, such as insulin dependent diabetes mellitus, multiple sclerosis, RA, and uveitis. We are counting on the BJYGC to be able to control the tendency of Th1 cell predominancy in an immune reaction.

Key words : Bojungykgitang-chunbang(BJYGC), IL-4, IFN-γ, polarization

Introduction

Immunity is somewhat of an instrument that a body uses as a mean of identifying its' self from non-self. It recognizes tissues of another cell or needless products in our body system as a non-self and eliminates them, therefore maintaining the body's homeostasis[1].

In Oriental medicine, immune deficiency is considered to be a factor of illness, thus defined 'weakness of primordial Qi'. Illness outbreaks when the harmony between one's internal environment and the outer environment gives away, consequently allowing invasion of pathogenic factors[2].

In western medicine, it is known that the T-cell is a decisive factor in immune system. The destiny of T cells is determined by contact with their cognate antigen bound to self major histocompatibility complex(MHC) on antigen presenting cells(APC)[3]. In the course of activation, cytokines produced by CD4 T helper (Th) cells divide their immune responses[4].

Naive CD4+ T cells combine with either MHC I or MHC II of the APC and differentiate into Th1 lymphocyte or Th2 lymphocyte. These two lymphocytes maintain the immune system through antagonism[5]. IFN-γ that is secreted from Th1 cell is known to restrain proliferation of Th2 cells by inducing the Naive CD4+ cells to differentiate into Th1 cells, On the other hand, IL-4 and IL-10 that are secreted from Th2 cells restrain Th1 cell's functions, by triggering the differentiation of Naive CD4+ T cell into Th2 cells[6]. These distinct subsets of Helper T cell are responsible for specific immune function. Th1 cells contribute to cell-mediated inflammatory immunity, while Th2 cells are responsible for humoral responses[7-8].

Actually, the regulation occurs in response to numerous environmental factors that directly or indirectly influence the decision of a naive CD4+ T cell to become a Th1/or Th2 effector cell and are occurred to immune disorder such as autoimmune disease and allergies, respectively[9]. In terms of

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· Received : 2004/01/26 · Revised :2004/02/27 · Accepted : 2004/03/25

pathology, Th1 predominance is observed in organ-specific autoimmune disease, whereas Th2 response is implicated in allergies[10]. Consequently, the key point in treating organ-specific autoimmune diseases or allergic diseases is regaining the balance lost in subsets of helper T cells.

As a kind of immunotherapy, the Oriental medicine presents a way to reinforce the body resistance in order to eliminate pathogenic factors[11]. The concept " immune " can be found in three of our organs, lung, spleen and kidney, based on theory of essential energy. The spleen, as an acquired energy, materially supports the essential and the defensive energy of our body. Any damage of spleen will cause deficiency of primordial energy, inevitably expanding its' chances of being a factor to all diseases[12]. All through the past years, there has been many studies on the effect of Bojungykgitang[12-13], Chungsangbohatang[5,14], and Sochungyongtang[15] on the activation of Th1/ Th2 cells. And without doubt, the effect of Bojungykgitang was the most outstanding of all.

Bojungykgitangchunbang(BJYGC)[16] is often clinically used as a treatment of allergic rhinitis. This is a prescription consisting of a few herbs with dispelling characters on top of the original Bojungykgitang. Bojungykgitang itself revives the energy of spleen and stomach, enhancing the body resistance. While the added herbs take charge of driving the pathogenic factors away, out of our body. The helper T cell take the most important part in immune system. We have experimented on the effect BJYGC would have on the helper T cell, and how it can promote the subsets of helper T cells to regain their balance that they lost due to immunological diseases.

Materials and Methods

1. Mice

Male BALB/c mice at 8 weeks of age were purchased from Samtaco, Korea.

2. Preparation of sample

The Sample(BJYGC) consists of Astragali radix[17-18] 6 g, Ginseng radix 4 g, Atractylodis macrocephalae phizoma 4 g, Glycyrrhizae radix 4 g, Angelicae gigantis 2 g, Citri pericarpium 2 g, Cimicifugae rhizoma 1.2 g, Cdidii rhizoma 4 g, Ledebouriellae radix 4 g, Bupleuri radix 2.4 g, Schizonepetae herba 4 g, Perillae folium 4 g, Menthae herba 2.4 g. A total of 226 g was extracted with water overnight and the supernatant collected on extraction was concentrated at 60 ℃ and evaporated to dryness in vacuo. 10.1 g (4.46 %) of extract powder was obtained. The sample was dissolved in PBS and sterilized BJYGC passing through 0.22 -㎛syringe filter.

3. Cell Purification and culture

Splenocytes were prepared from BALB/c mice and treated with red blood cell lysing buffer (BD Pharmingen, U.S.) CD4 T cells were magnetically isolated by using magnetic microbead-conjugated anti-mouse CD4 mAbs (L3T4) (Miltenyi, Germany), as described by the manufacturer. In brief, CD4 T cells were separated by passing the cell suspension over a magnetic-activated cell sorter(MACS) MS+ column held in MACS magnetic separator. The CD4 T cells adhering to the column were cultured in RPMI medium containing 10 %.Fetal bovine serum(FBS). Cells were stimulated with or without immobilized anti-CD3/CD28 Ab (Pharmingen) and incubated for 48 hr at 37 ℃ in 5 % CO_2 with BJYGC at various concentrations.

4. Viability and Proliferation assay

To measure the viability and proliferation capacity of CD4 T cells in the presence of BJYGC, cells were placed in a 96 well for 48 hr and then treated with the tetrazolium salt MTS{3-(4,5-dimethylthiazol-2-yl)-5-(3carboxymethoxyphenyl)-2H -tetrazolium} (Promega, U.S.)

5. Analysis of cell surface expression

For cell surface staining, cells were cultured in 24-well for 48 hr. Staining for cell surface markers was performed as described by the manufacturer's instruction. In brief, cells were harvested and centrifuged 1000 rpm for 5 min. After removing supernatant, the pellet was resuspended in cold wash buffer (PBS/ 0.1 % NaN3/ 1 % FBS), centrifuged and resuspended in 100 ㎕ of wash buffer and stained with Fluorescein isothiocyanate (FITC)-conjugated CD25 (Pharmingen) and incubated at 4 ℃ in the dark for 40 min. After washing twice, the cells were analyzed with a Becton Dickinson FACScan.

6. Th1/2 cell polarization

CD4 T cells were stimulated with anti-CD3/CD28 Ab plus recombinant IL-2 for 3 days. For Th1 polarizing condition, rIL-12 and anti-IL4 Ab were added and for Th2 polarizing condition, cells were added with rIL-4 and anti-IL12 Ab.

7. Measurement of cytokine production by CD4 T cells.

The levels of IL-4 and IFN-γ in the culture supernatant were measured with BD OptEIA Mouse IL-2, IL-4 and IFN-γ set (Pharmingen). The assay sensitivities were 200- 3.1 pg/ml (IL-2), 31.25-2000 pg/ml (IFN-γ) and 7.81-500 pg/ml (IL-4). The plates were read at 450-570 nm and the sample concentrations were determined with the help of the standard curve.

8. Statistical analysis

All the data expressed are mean numbers with an acceptable error range depending on the statistical difference (mean ± S.D). Independent T tests were applied in order to determine the statistical differences between the groups.

Results

1. The effect of BJYGC on the viability and proliferation of CD4 T cells

The viability of CD4 T cells from Balb/c mouse were measured at various concentrations of BJYGC using the MTS assay. Up to 400 ㎍/㎖ it slightly increased survival of CD4 T cells (Fig. 1). However, when cells were activated with anti-CD3/CD28 at the same range of concentration for 48 hr, the peak concentration was 10 ㎍/㎖ with a 40% increase and there was a moderate increase in higher concentrations (Fig. 2).

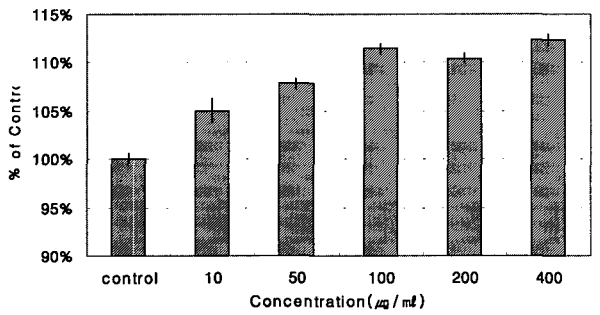

Fig. 1 The viability of CD4 Th cells treated with BJYGC. CD4 T helper cells from Balb/c mouse were purified by magnetic cell sorting and cultured in the presence of BJYGC for 48 hr. Cell survival test was measured using the MTS assay.

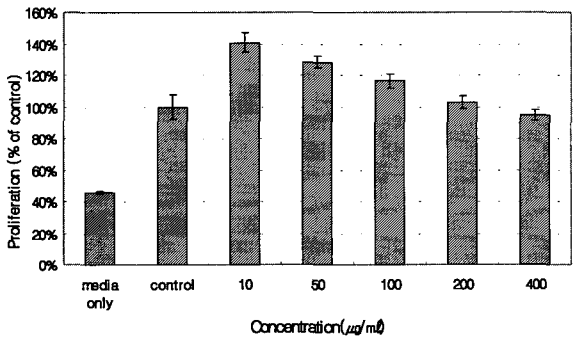

Fig. 2. CD4 Th cells were stimulated with anti-CD3/28 in the presence of BJYGC for 48 hr. Proliferation was measured using the MTS assay.

2. The effect of BJYGC on CD25 expression of CD4 T cells

As evidence for rapid T cell activation, CD25 expression by flow cytometry was evaluated. CD25, which is identified as the alpha chain of the high-affinity interleukin-2(IL-2) recepotr, is considered an early activation marker of T cell. At 10, 50, 100 and 200 ㎍/㎖ of BJYGC, there was no remarkable difference in CD25 expression (Fig.3). Although it increases cell proliferation, BJYGC showed a little effect on the qualitative response of CD4 T cells, making IL-2 less effective for lymphocyte proliferation.

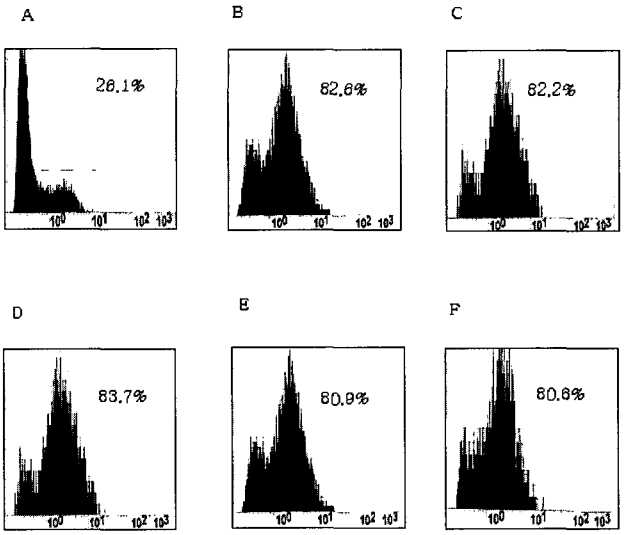

Fig. 3. Expression of CD25 on BJYGC treated CD4 T cells. CD4 Th cells were stimulated with anti-CD3/28 in the presence of BJYGC. 48 hr later cells were stained with FITC-conjugated anti-CD25 and analyzed by flowcytometry. A: cells in media alone. B: cells stimulated with anti-CD3/28. C-F: cells stimulated with anti-CD3/28 in the presence of BJYGC 10, 50, 100 and 200 ㎍/㎖.

3. The effect of BJYGC on Th cells' differentiation

Th cell differentiation experiments were performed to examine whether BJYGC can affect the Th polarization process. CD4 T cells were activated in culture under neutral, Th1-polarized or Th2-polarized conditions in the presence of BJYGC at 10, 100 and 200 ㎍/㎖. Supernatants were collected and the production of IFN-γ and IL-4, the signature cytokines for Th1 and Th2 cells, respectively, was measured by an ELISA.

In the case of CD4 T cells under neutral condition where there was only rIL-2 stimulus, 100 and 200 ㎍/㎖ of BJYGC inhibited IFN-γ secretion BJYGC 90 % (Fig. 4) Likewise, BJYGC also inhibited the IFN-γ secretion of Th1 polarized cells(Fig. 5); 10 ㎍/㎖ of BJYGC decreased IFN-γ by 40 % and higher concentrations showed stronger inhibition.

Similar results were obtained from the IL-4 production of both the neutral and Th2 polarized cells. Although the IL-4 production at 10 ㎍/㎖ of BJYGC was slightly increased in neutral culture and increased in Th2 polarized cells, 100 and 200 ㎍/㎖ of BJYGC inbibited the IL-4 production of the neutral Th cells and Th2 cell by 80 % and 50 %, respectively.

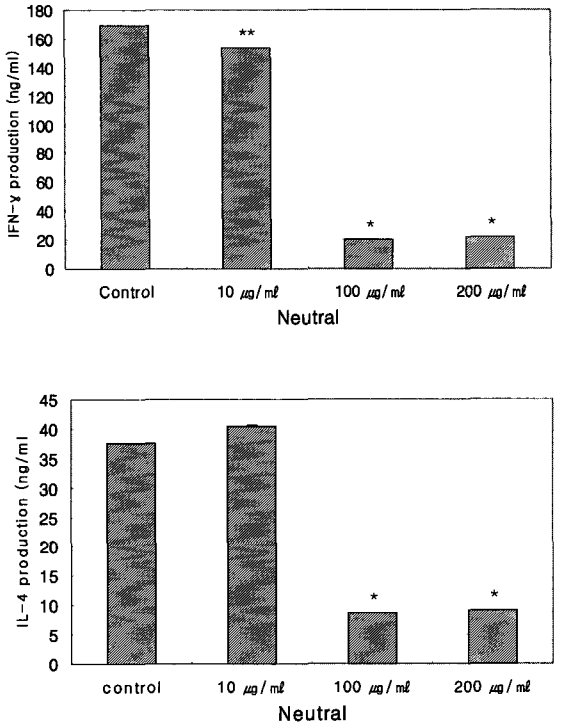

Fig. 4. Effect of BJYGC on Th1/2 cytokine profiles of murine CD4 T cells. CD4 T cells were activated in vitro under neutral condition in the presence of BJYGC for 72 hr. Cytokine production was measured by ELISA. *: significant at 0.001, **: significant at 0.005.

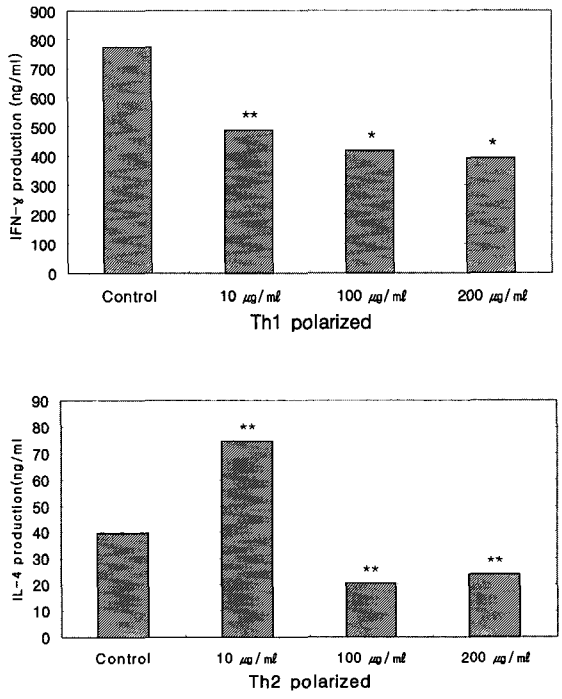

Fig. 5. Effect of BJYGC on Th1/2 cytokine profiles of murine CD4 T cells. CD4 T cells were activated in vitro under Th1 and Th2 polarizing conditions in the presence of BJYGC for 72 hr. Cytokine production was measured by ELISA. *: significant at 0.001, **: significant at 0.005.

Discussion

The discovery of dichotomy between Th1 and Th2 helper cells represents one of the most important advances in immunology. The Th1/Th2 concepts suggests that modulation of relative contribution of Th1 or Th2 type cytokines regulate the balance between protection and immunopathology, as well as the development and severity of some immunologic disorders. Both Th1 and Th2 subsets generate from the same precursor, the naive CD4+ T lymphocytes. The form of differentiation is determined by the existing stimulation during the initial immune reactions. The most important inducer in stimulating the differentiation is cytokine. IL-12 is the main inducer of Th1 cell and IL-4 is that of Th2 cell. Th1 cell secretes IL-2, IFN-γ and so forth, while Th2 cell secretes IL-4, IL-10 etc. Interleukin-4(IL-4) production by a still unknown cell type at the time of antigen presentation to the Th cell is critical for the development of Th2 cells. Other cytokines, such as IL-1 and IL-10, and hormones, such as calcitriol and progesterone, also play a favoring role. In contrast, cytokines such as interferon (IFN-α, IFN-γ), IL-12 and transforming growth factor (TGF)-beta, and hormones, play a negative regulatory role on the development of Th2 cells[19].

Evidence has accumulated from animal models to suggest that Th1 type lymphokines are involved in the genesis of organ-specific autoimmune diseases, such as experimental autoimmune uveitis, experimental allergic encephalomyelitis, or insulin-dependent diabetes mellitus. By contrast, Th2 cell predominance was found in the skin of patients with chronic graft-versus host disease, progressive systemic sclerosis, systemic lupus erythematosus, and allergic diseases[20]. Analysis of Th subsets at different intervals after allergen challenge showed that Th2 cell play an important role in initial phase of inflammatory reactions whereas in later stages Th1 cells can be detected in greater numbers[20].

Spleen and stomach takes an important role in the defence mechanism of our body. It is because spleen and stomach materially supports the essence and defensive energy of our body, based on their acquired foundation of energy. Bojungykgitang remedies the deficiency of Qi in the middle, strengthening the Qi of stomach and spleen, therefore being a very useful prescription in enhancing the immune function. Also, Jeong[21] emphasized the importance of using a treatment theory of releasing the exterior in order to dispel the pathogenic factor in immune disorders like allergic disease. Their article, 'Approaching allergic diseases from the oriental medical perspective', mentions as follows, "Antigens can be classified into various types according to the different

pathogens, such as wind, coldness, heat, dryness, or humidity.

Pathological changes also appear differently in each cases. In cases of allergies, the symptoms are superficial, and is, what we call the Yang syndrome. So prescriptions used widely for Taeyang diseases, with dispelling natured herbs like Cinnamomi Ramulus and Ephedrae Herband, are recommended. "Hereupon, this writer performed an experiment on herbs in BJYGC, of which the effect is based on this theory. They are Cdidii rhizomag, Ledebouriellae radix, Bupleuri radix, Schizonepetae herba, Perillae folium and Menthae herba, and they were added to the original Bojungykgitang, completing the BJYGC. The purpose of this experiment was to find out if the BJYGC could help regain an balance of Helper T cell subsets.

First, in order to verify if the BJYGC extract stimulates splenocyte as a mitogen, splenocytes were treated with gradational concentration of the extract and cultured for 48 hours. Then the viability was measured by MTS assay. It was somewhat increased up to concentration of 400 μg/ml, but did not show any significant difference.(Fig.1) This means that the extract does not stimulate splenocytes as a mitogen. When there was an additional stimulation of anti-CD3/28 on CD4 Th cell, in the same condition of time and concentration as above, the proliferation increased up by 40 % at concentration of 10 μg/ml.(Fig.2) Expression of CD25 was measured, with stimulation of anti-CD3/28 in the presence of BJYGC. It turned out that gradational concentration of BJYGC treatment did not affect T cell's activity.(Fig.3) In neutral state, secretion of IFN-γ decreased down by 90 % with BJYGC at concentration of 100, 200 μg/ml. IL-4 increased a bit at 10 μg/ml, but then again decreased down by 80 % at 100, 200 μg/ml of BJYGC.(Fig.4) In polarized state, the secretion of IFN-γ decreased by 40 % with 10 μg/ml of BJYGC and even more, proportional to the concentration of BJYGC. IL-4 was increased a bit at the concentration of 10 μg/ml, but dropped down to 50 % at 100, 200 μg/ml.(Fig.5) This experiment proved that BJYGC could inhibit the secretion of both IL-4 and IFN-γ in neutral condition and polarized condition, too.

It is suggested that BJYGC may exert some action on intracellular or extracellular IFN-γ production depending on the concentration of IFN-γ in the surrounding. Further studies are needed to investigate in what pathway BJYGC takes part. IL-4 secreted from Th2 cell activates B cell and generates IgE. IgE combines with the antigen, causing degranulation of a mast cell, consequently releasing chemical substances such as histamine, serotonine and etc. This brings on smooth muscle contraction, myxedema and vasodilation which ultimately leads to diseases like asthma or allergic rhinitis[22-23]. BJYGC in the current study demonstrated suppressing effects on IL-4 production in both neutral and Th2 polarizing conditions.

Considering that BJYGC shows an excellent effect on treating allergies, the author can conclude that its pharmacological action may be associated with decreased IL-4 and, it may also regulate IFN-γ depending the host's need. Also, it was discovered that Th1 cell was pathologic in chronic inflammatory tissue specific diseases, such as insulin dependent diabetes mellitus, multiple sclerosis, RA, and uveitis. We are counting on the BJYGC to be able to control the tendency of Th1 cell predominancy in an immune reaction.

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