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원 저

The Experimental study of *Jungchun-tang* on Allergies

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정천탕의 알러지에 대한 실험적 연구

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목적 : 정천탕(定喘湯, JCT)이 IgE/mast cell로 유도되는 천식(喘息)등의 알러지성 질환에 대한 효과를 실험적으로 검증하고자 하였다.

방법 : IgE의 혈중농도와(in vivo) 분비량을 측정하였으며(in vitro), 알러지 관련 유전자의 발현을 RT-PCR로 검색하였고, JCT의 수용성 복합 추출물이 compound 48/80로 유발되는 mast cell line(IC-2)의 histamine의 방출에 미치는 직접적인 영향을 측정하였다(in vitro).

결과 : 정천탕은 ovalbumin으로 유발시킨 마우스의 혈중 IgE량과 IL-4와 CD-40로 유도시킨 spleen B 임파구의 IgE 생성량도 현저하게 감소시켰다. 또한 INF-r의 발현은 증가시킨 반면, IL-4와 IL-6의 발현과 mast cell의 histamine 방출은 억제하는 것으로 나타났다.

결론 : 정천탕의 항알러지 효과를 실험적으로 확인할 수 있었으며 임상적으로는 천식 등 알러지성질환에 사용할 수 있을 것으로 사료된다. *(J Korean Oriental Med 2003;24(3):65-71)*

Key Words: *Jungchun-tang*, IL-4, IL-6, anti CD40, IgE Production, Histamine

Introduction

When an adaptive immune response occurs in an exaggerated or inappropriate form, the term *hypersensitivity* is applied[1]. *Allergy* refers to certain diseases in which immune responses to environmental antigens cause tissue inflammation and organ dysfunction. Three different allergies according to the pathways of immunologically induced inflammation are:

(A) the IgE/mast cell/mediator pathway,

(B) the IgG or IgM immune complex/complement/ neutrophil pathway, and

(C) the effector T-lymphocyte/ lymphokine pathway[2].

Hay fever, asthma, urticaria, or chronic eczema are the most common atopic diseases following exposure to environmental allergens such as pollen, house dust

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mites, mold, animal dander or foods and induced by the IgE/mast cell/mediator pathway, called immediate hypersensitivity[3]. These are worldwide diseases, usually affecting 10-30% of all individuals in developed countries[4].

The mainstay of therapy for atopic disorders is to block the production or release of mediators, or to antagonize mediator actions on target cells, and to diminish synthesis of IgE. In contrast to healthy individuals, allergic patients show elevated total serum IgE level and develop specific IgE directed against sensitizing allergens that play a key role in the pathophysiology of allergic disease[5,6]. In addition, these individuals have more high-affinity Fcε receptors on each mast cell, and a larger proportion of these receptors are occupied by IgE compared with those in non-atopic individuals[5].

Jungchun-tang (JCT) is a traditional herbal-prescription for patients suffering from asthma and bronchitis in oriental medicine. In the present study the effects of JCT on allergy were investigated by quantifying the IgE level in vivo and in vitro, histamine release in vitro, and allergy-related cytokine gene expression with RT-PCR. The resulting data suggest that JCT have effects involved with IgE synthesis by controlling allergy-related cytokine and reducing histamine release from mast cells.

Materials and Methods

1. Plant materials

JCT' s prescription is as fallow: Puerariae Radix, Cinnamomi Ramulus, Zingiberis Rhizoma, Scrophularie Radix, Moutan Cortex Radicis, Scutellariae Radix, Schizandrae Fructus. These plants were boiled in water for 2 hours and a soluble extract was obtained through evaporator and freeze dryer. Proper concentrations were made for administration to animals with water and for treatment to cells with medium.

2. Animals

Male C56/BL6 mice were purchased from a commercial animal breeder (Dae-Han Laboratory Animal Research Center, Korea). Mice were divided into four groups:

Normal, Control (water), JCT I (100mg/kg), and JCT II (300mg/kg), each consisting of 8 animals. JCT solved in water was administrated orally every day, while the control group was administrated just water.

3. IgE induction assay in vivo

Ovalbumin (1mg/ml [Sigma], USA) and equivalent volume of FCA (Sigma, USA) were emulsified, then subcutaneously injected to C57BL/6 mice (0.1ml/10g) on the first day and another week after. At 1 week after the second immunization, mice were sacrificed and total serums were obtained. The serum total IgE level was measured by using indirect ELISA kit (Pharmingen, USA)7)

4. Anti-CD40 + IL4 -mediated IgE production in vitro

BALB/c mice spleens were removed and treated with anti-Thy1.2 (200 ul/108 cells, Pharmingen, USA) in ice for a half-hour, followed by twice washing with D-PBS.

After 37° C water incubation with 0.5ml of rabbit complement lyophilised (Serotec, U.K.) and washed 5 times, pure spleen B cell isolated was passed through Sephadex G-10 column (Amersham Pharmacia, USA). With a little modification of the method described by Armitage RJ8), spleen B cells were cultured in 24 well plates (2 × 106 cells/well) with triplete per every group with various concentrations of JCT (1ug/ml, 10ug/ml, 100ug/ml) and anti-CD40 (100ng/ml, Pharmagen, USA) IL-4 (500 U/ml, Pharmagen, USA) in 37° C, 5% CO2 incubator. After 14 days incubation, cells were

centrifuged (2,000 rpm) to collect cell-free supernatant and released IgE was measured with indirect ELISA kit (Pharmingen, USA).

5. Allergy-related cytokine gene analysis with RT-PCR

Pure B cells were isolated through the same process described above in anti-CD40 + IL4 -mediated IgE production in vitro. B cells (1x106 cell/well) in 24 well plates were precultured with JCT (1ug/ml, 10ug/ml, 100ug/ml) for 1 hour. After treatment with anti-CD40 mAb (100 ng/ml) and rmIL-4 (500 U/ml) for 6 hours, cells were harvested and other processes (total RNA isolation, reverse transcription, cDNA-PCR) were done according to general RT-PCR method. Sequences of the deoxynucleoted for PCR are as follows: IL-4: 5' -ATGAACTCCTTCTCCACAAGCGC-3' and 5' -GAAGAGCCCTCAGGCTG GACTG-3' , IL-1β: 5' -CCTCTTCTTGAGCTTGCAAC-3' and 5' -AGCCCATGAGTTCCAT TCAC-3' , IL-6: 5' -CCGTCGATAGTGGCATCCATGAAAC-3' and 5' -GGACCAATACCT GCTATAGGG-3' , INF-r; 5' -AGCGGCTGACTGAACTCAGTGTAG-3' and 5' -GTCACAGTTTTCAGCTGTATAGGG-3, β-actin: 5' -TGGAATCCTGATCCATGAAC-3' and 5' -TAAA ACGCAGCTCAGTAGTCCG-3' .

PCR products were measured as height value by using Windows ID main program (AAB, USA).

6. Compound 48/80-induced histamine release in vitro

IC-2 mast cells were cultured in 24 well plates (2× 105 cells/well) with RPMI1640 containing rIL-3 (10uU/ml R&D system, USA) and various concentrations of JCT (1ug, 10ug, 50ug, 100ug, 500ug, 1000ug/ml) for 30 minutes. After treatment with compound 48/80 (5ug/ml) for 20 minutes, supernatant was obtained and released histamine was measured with commercial kit (Immuno Tech., France). The inhibition percentage of histamine release was calculated by using the following equation:

$$\frac{\text{(histamine release without JCT - histamine release with JCT)}}{\text{histamine release without JCT 100}}$$

7. Statistical analysis

The results obtained were expressed as mean±SD for the number of experiments. Student' s *t*-test was used to make a statistical comparison between the groups. Results with $p<0.05$ were considered statistically significant.

Results

1. IgE induction assay in vivo

After obtaining the serum, total serum IgE concentration of each group (n=8) was measured with quantitative analysis by using commercial ELISA kit (Pharmingen, USA). In brief, ELISA was performed by coating 96 well plates with anti-IgE Ab at 4° C overnight and washing 3 times followed by blocking and washing process again. After adding 100ug of diluted serum (50:1) the plate was incubated at room temperature for 2 hours and washed 5 times. After adding detection Ab, the plate was incubated at room temperature for 1 hour and washed 7 times. 100ul of TMB substrate solution was added for 30 minutes and IgE concentration was determined by reading the colormetric absorbent with 40nm and comparing the value with standard value.

As shown in Fig. 1, JCT-treated groups (100 and 300mg/kg) presented lower total IgE concentration than the control group with significance ($p<0.01$), but the results were not relative to JCT-dose.

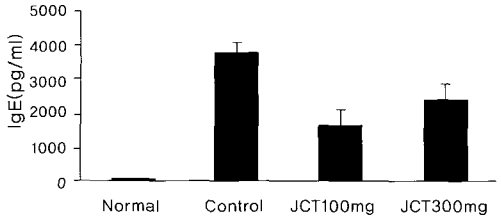

Fig. 1. Effect of JCT on inhibition of IgE production in vivo. C57BL/6 mice (n=8) were immunized with ovalbumin (s.c. 0.1ml/10g in 1mg/ml normal saline and FCA) on first and 8th day and were administered JCT (p.o.) for 14 days. The serum total IgE level was measured on the last day.

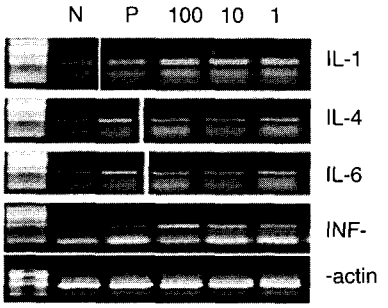

Fig. 3. Analysis of cytokine gene expression after JCT treatment. Spleen B cells were cultured in 24 well plates (1×106 cells/well) with anti-CD40 (100ng/ml) L-4 (500 U/ml) for 6 hours after JCT (1ug/ml, 10ug/ml, 100ug/ml) preculture for 1 hour. N and P represent negative control (only medium) and positive control (anti-CD40 +L-4).

2. Anti-CD40 + IL4 -mediated IgE production in vitro

After 48 hours incubation, B cell-synthesized IgE was measured using the commercial ELISA kit (Pharmingen, USA) and same process done in IgE induction assay in vivo. As shown in Fig. 2, JCT treatment inhibited the IgE synthesis from spleen B cells which were activated with anti-CD40 + IL4-mediated IgE to differentiate to IgE releasing plasma cells with significance ($p<0.01$).

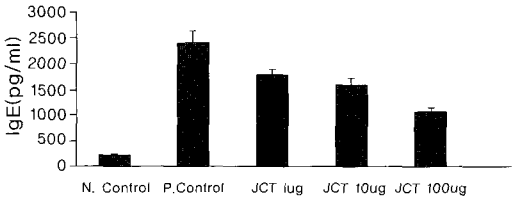

Fig. 2. Effect of JCT on inhibition of IgE production in vitro. Spleen B cells were cultured in 24 well plates (2× 106 cells/well n=3) with various concentration of JCT (1ug/ml, 10ug/ml, 100ug/ml) and anti-CD40 (100ng/ml) L-4 (500 U/ml). After 14 days incubation, released IgE was measured in cell-free supernatant.

3. Analysis of RT-PCR

IL-4, IL-1, IL-6 and INF-r mRNA were analyzed by RT-PCR after treatment with anti-CD40 mAb (100 ng/ml) and rmIL-4 (500 U/ml) or JCT (1ug/ml, 10ug/ml, 100ug/ml) for 6 hours. The results showed that JCT had little effect on inhibition of IL-4, IL-6 mRNA gene expression, then IL-1and INF-r mRNA expression was decreased by adding JCT as in Fig. 3.

4. Inhibition of Compound 48/80-induced histamine release

The inhibitory effects of JCT on compound 48/80-induced histamine release from IC-2 mast cells are shown in Fig. 4, 5. JCT inhibited the histamine release from IC-2 mast cells which were activated by histamine released agent, compound 48/80 (5ug/ml), depending on the concentration of JCT.

Discussion

Allergy resulting from exposure to nontoxical antigen is a significant environmental and occupational health problem. In general, immediated hypersensitivity, which involves urticaria, allergic rhinitis and asthma, is brought through the IgE/mast cell/mediator pathway

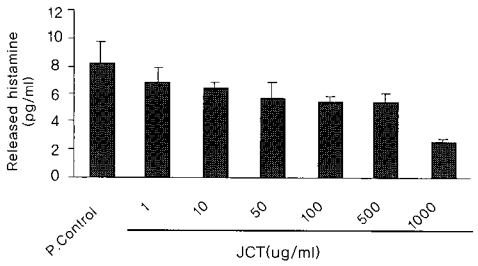

Fig. 4. Effect of JCT on inhibition of histamine release from IC-2 cell with compound 48/80 in vitro. IC-2 mast cells were cultured in 24 well plates (2 × 105 cells/well) with RPMI1640 containing rIL-3 (10uU/ml) and various concentrations of JCT for 30 minutes. After treatment with compound 48/80 (5ug/ml) for 20 minutes, supernatant was obtained and released histamine was measured with a commercial kit.

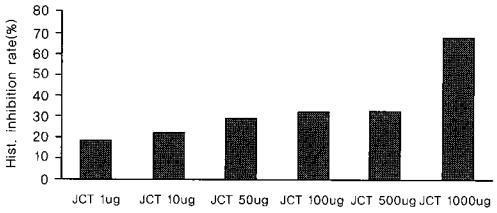

Fig. 5. Effect of JCT on inhibition rate (%) of histamine release from IC-2 cells with compound 48/80 in vitro. The inhibition percentage of histamine release was calculated by using the following equation: (histamine release without JCT - histamine release with JCT) / histamine release without JCT 100.

and is most common among the three types of allergies. This disease is related with high level serum IgE concentration or high-affinity Fcε receptors on each mast cell[5] and is mediated by various chemical mediators released from mast cells[3].

The present study showed that JCT inhibited the production of IgE in mice model induced by ovalbumin (Fig. 1) and anti-CD40 + IL4 -mediated IgE production in vitro (Fig. 2). It has been well established that ovalbumin elicits IgE production in BALB/c strain mice[9] through preferential Th2-type responses, where the predominance of IL-4 over IFN-production would be permissive for IgE responses[10]. It is well known that in mice the integrity of IgE antibody production is regulated by cytokines. The initiation and maintenance of IgE response are dependent upon the availability of IL-4[11,12], whereas another cytokine, IFN- antagonizes IgE antibody production[13]. These immunoregulatory cytokines are the products, respectively, of Th1and Th2 cells, these being subpopulations of CD4+ Th cells[14,15]. Then, we can suggest that JCT inhibits IgE production by regulation of cytokine expression, of course, where IFN- is predominant to IL-4 production as seen in Fig. 3.

Induction of IgE synthesis requires two signals and has been shown to be induced by the cytokine IL-4 and engagement of the B-cell antigen CD40[16]. CD40 is a surface Ag on B cells and stimulation of B cells via the CD40 molecule can induce a wide variety of effects on B cells, including growth, differentiation, and rescue from B cell Ag receptor-mediated apoptosis[17,18]. In combination with IL-4, stimulation of CD-4 leads to enhanced germline mRNA expression, functional gene transcription, and IgE secretion[19,20]. We experimented on the effects of JCT by detecting the IgE production from purified mouse spleen B cells induced by IL-4 and recombinant anti-CD40. A dose-dependent inhibition with JCT of IgE secretion from B cell was observed significantly. IL-4 is a class switching factor inducing expression of the germline transcript of the C gene, which maintains an open chromatin structure in this region.

The activation of CD40 results in the induction of the class switching machinery for S-S recombination[21]. CD40 + IL-4 mediated IgE production can be inhibited by INF- or other cytokine and various factors such as hormones[22]. It is strongly suggested that IL-6 is a necessary step in CD40-induced IgE production because of the results that neutralizing anti-IL-6 Ab

inhibited the ability of anti-CD40 Ab and IL-4 to induce IgE synthesis[23], and IL-1 or TNF- induced IL-6 gene expression and NF-B DNA binding activity which is related with regulation of IL-6 expression[24,25]. As seen in Fig. 3, JCT inhibited IL-4 A and IL-6 mRNA expression, but promoted IFN- expression, and didn' t show any effect to IL-1 gene expression. So, it is possible to hypothesize that JCT might act on cytokine expression such as IL-4, IL-6 and INF-, affecting the IgE production.

In the immediate hypersensitivity, mast cell-released mediators are essentially responsible for pathologic features, so histamine is most critical. It is well recognized that compound 48/80 causes a potent histamine release from mast cells by activating G proteins and is used as a histamine liberator for estimating the effectiveness of antiallergic drugs[26,27]. As seen in our study in Fig. 4, 5, JCT significantly inhibited the compound 48/80-induced histamine release from mast cell line, IC-2 cell. However, the dose effect of anti-degranulation from mast cells remains to be clearly determined.

In conclusion, the results obtained in this study provide evidence that JCT has effectiveness on immediate type allergic reaction by inhibiting IgE production with cytokine regulation and histamine release. To our knowledge, this is first report such of research for IgE/mast cell/mediator allergic disease by JCT.

Acknowledgements

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