In the present study, we investigated the immunoregulatory effects of *Stemona tuberosa* (ST) in an *in vitro* and in ovalbumin (OVA)-induced asthma model. Murine spleen cells were isolated from normal and OVA-treated mice and the cytokine levels from T-cells in supernatants were determined by enzyme-linked immunosorbent assay (ELISA). The results of the *in vitro* experiments indicated that ST modulated the balance between Th1 and Th2 cytokines. In addition, in the *in vivo* experiments we showed that ST significantly increased the levels of interleukin-2 (IL-2) and interferon-γ (IFN-γ) and decreased those of IL-4, IL-5 and IL-13, as compared with OVA control. Reverse transcription-polymerase chain reaction (RT-PCR) was carried out to determine the GATA-3/T-bet ratio. Results showed that ST modulated the Th2/Th1 imbalance by inhibiting GATA-3 and enhancing T-bet. The hematoxylin and eosin (H&E) stain studies on lung tissues indicated that ST had potential inhibitory ability on inflammation cells. Taken together, these findings suggest that the ethanol extract of ST might have compounds with immunomodulation potential.

**Key words:** *Stemona tuberosa*, immunoregulation, allergic asthma, T-cell cytokines.

**INTRODUCTION**

*Stemona* herb, called Baibu in Chinese and included in the People’s Republic of China Pharmacopoeia (Pharmacopoeia of PR China, 2010), has been widely used as an antitussive and anthelmintic in southern Chinese medicine for over 2000 years (Jiangsu New Medical College, 1977; Brem et al., 2002). Nowadays, extracts from its roots have been used to relieve respiratory disorders, including pulmonary tuberculosis, acute or chronic bronchitis and bronchial asthma, and has been intensively used as an anthelmintic and as an insecticide for enterobiasis and lice (Cong and Xu, 1997; Philli and Ferreira, 2000; Xu, 2000). Researches on antibacterial activities (Zhao et al., 1995, Yang et al., 2006), antitussive functions (Xua et al., 2006) and insecticidal behavior (Kalteneggera et al., 2003) of extracts from this herb were developed in the last 15 years. However, there is no scientific report on the immunoregulatory effects of this herb. Alterations of the immune system can lead to illness and infection. Asthma is a prevalent and serious chronic inflammatory airway disease that affects 300 million people worldwide, including both adults and children, and an acute asthma attack may be fatal (Cohn et al., 2004).

Over the last decade, investigations into the pathogenesis of asthma have focused on the balance of Th1, Th2 and recently Th17, in asthmatics and animal models (Majdalawieh et al., 2010; Alcorn et al., 2010).
Th17 cells secrete interleukin (IL) IL-17, IL-17F, IL-6, IL-22 and Tumor necrosis factor-alpha (TNF-α) and appear to play an integral role in tissue inflammation and activation of neutrophils to attack extracellular bacteria (Kaiho et al., 2008). Th1 cells are mainly involved in cell-mediated immune responses mainly mediated by interferon-γ (IFN-γ), IL-2 and IL-12. Moreover, Th1 cytokines inhibit eosinophil infiltration, immunoglobulin E (IgE) and IgG1 production, but stimulate IgG2a production in vivo. In particular, IFN-γ may act as an inhibitor of the Th2 pathway (Morokata et al., 1999). Similarly, Th2 cells are involved in humoral immune responses mediated by granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-4, IL-5, IL-6, IL-9, IL-10, IL-13 (Ogawara et al., 2005; Romagnani, 2000). IL-4, IL-5 and IL-13 are the main cytokines in the asthmatics models for IgE synthesis, airway eosinophilia, mucus secretion, and ultimately airway hyper-responsiveness (AHR) (Busse and Lemanske, 2001; Ngoc et al., 2005). IL-4 is known to cause B-cells antibody switching, resulting in IgE synthesis (Fish et al., 2005; IL-5 plays a key role in enhancing eosinophil recruitment, activation, and survival at inflammation sites (Takatsu and Nakajima, 2008); IL-13 plays a significant role in the effector phase of Th2 responses, such as eosiinophilic inflammation, mucus secretion and AHR (Wynn, 2003). The levels of these cytokine result in the imbalance between Th1 and 2 responses. A greater abundant presence of Th2 cells and their relative cytokines and a decrease of Th1 cells and their relative cytokines are markers of the presence of the disease, thus, a way to cure asthma focuses on modulating the levels of these cytokines (Gariboldi et al., 2009). Although regulation of the Th1/Th2 imbalance remains unclear, and the differentiation of T-helper cells is sophisticatedly controlled, growing evidence suggests that T-bet and GATA-3 (GATA-binding protein 3) are 2 major T-helper-specific transcription factors that play a key role in Th cells differentiation (Yu et al., 2003). T-bet as T-box expressed in Th cells is a newly discovered Th1-specific transcription factor that may initiate Th1 development and inhibit Th2 differentiation. In contrast, GATA-3 promotes the development of the Th2 phenotype and inhibits Th1 differentiation. The balance of Th1/Th2 can be expressed by the ratio of T-bet/GATA-3 (Chakir et al., 2003). Cyclooxygenase 2 (COX-2) is a key factor for the progression of inflammation (Kiefer and Dannhardt, 2002; Stichenon and Frolich, 2003), and several clinical diseases (Koki et al., 2002; Menter 2002). It has been reported that the COX-2 inhibitor alters Th1/Th2 cytokine balance and inhibits cell proliferation in vitro (Suzuki et al., 2006).

Therefore, the objective of the present work was focused on identification of immunoregulatory effects of Stemona tuberosa (ST) in vitro and in an ovalbumin (OVA)-induced mouse asthma model by determining T-cells cytokines, and reverse transcription-polymerase chain reaction (RT-PCR) production of T-bet, GATA-3 and COX-2.

MATERIALS AND METHODS

Identification and preparation of ST roots and extracts

ST roots were purchased from Haozhuo drugs market, Anhui province of China. They were cut into 0.5 cm length pieces and fixed in Formalin:glacial acetic acid (FAA): 70% ethanol = 5:5:90 (v:v:v). Fifteen micrometer (15 µm) transverse sections of each sample were cut using a Leica CM1850-1-1 autocut automatic microtome (Leica Microsystems Nussloch GmbH, Germany) at -20°C. One to two drops of Sudan III solution were added to sections at room temperature, allowed to stand for 3 min, and then rapidly heated to boiling. Dilute glycerin was used to wash away excess of Sudan III. Ten slides of each prepared sample were then made. To prepare ST extract, roots were powdered in a grinder and 1 kg of the powder was extracted with ethanol (1:10, w/v) at 20°C for 7 days. The extracts were filtered, and the filtrates were concentrated by evaporation under reduced pressure at 45°C to produce 101.6 g of dry extract named ST, which was stored at 4°C before use. ST was dissolved in 1% tween 80 to different concentrations for oral administration in mice. For cells, ST was dissolved with 1% dimethyl sulfoxide (DMSO) to different concentrations.

Animals

Male 6 to 8 weeks old BALB/c mice, weighting between 18 and 20 g, were purchased from the Experimental Animal Center of Dalian Medical University (Dalian, China. Quality Certificate Number: SXCK (L) 2008-0002) and received care according to the National Institutes of Health guidelines.

Reagents

Concanavalin A (ConA), OVA, hematoyxin and eosin (H&E), RPMI-1640, heat-inactivated fetal bovine serum (FBS), penicillin-streptomycin, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), and other tissue culture reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA), enzyme-linked immunosorbent assay (ELISA) kits for IL-2, IL-4, IL-5, IL-13 and IFN-γ were obtained from R&D system (USA), Trizol was bought from Invitrogen (USA), RT and PCR kits were obtained from Thermo system (USA). All primers were synthesized by Saibaiheng company in Beijing. Other chemical reagents were obtained from Kernel company (Tianjin).

Sensitization, challenge and treatment

Mice received the following treatments: Phosphate buffered saline (PBS) control group (n = 8); sham-sensitization plus challenge with PBS; OVA group (n = 10): sensitization plus challenge with OVA; Treated group (n = 10): sensitization with OVA (intraperitoneal; i.p.) plus challenge with OVA and ST (500, 250 or 125 mg/kg/day). In brief, mice were sensitized by i.p. injection of either PBS or 20 μg OVA emulsified in 2 mg aluminum hydroxide in 200 μl PBS buffer (pH 7.4), on days 0 and 14. On days 18 to 31, ST was administered
Figure 1. Schedule of mouse model of airway inflammation and treatment with S. tuberosa.

Isolation of splenocytes

Spleen cells were obtained from normal and treated mice with PBS or OVA, respectively. In brief, spleens were taken out under axenic conditions, and lysed using ACK lysis buffer (0.15 M NH₄Cl, 1 mM KHCO₃, and 0.1 mM Na₂EDTA), then resuspended in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, and 1% penicillin-streptomycin. Cells viability was measured by trypan blue exclusion technique (>98% viability was observed).

MTT assay

Spleen cells from normal mice were cultured in flat-bottomed 96-well plates, adjusted to 1 × 10⁶ cells/mL, and cultured for 48 h at 37°C. Twenty microliters (20 µl) of ST at the concentrations of 8, 4, 2 and 1 mg/mL, plus 20 µl ConA (50 µg/mL) were added to wells (three wells for each experiment), the volume was adjusted to 200 µl with RPMI 1640. Cells were incubated for another 72 h. DMSO with a final concentration of 0.1%, instead of ST in the culture medium was set as control group. The mitochondrial-dependent reduction of MTT to formazan was used to measure cell respiration as an indicator of cell viability (Denizot and Lang, 1986). Briefly, a MTT solution (final concentration is 500 µg/mL) was added and cells were incubated for another 4 h at 37°C. After removing the supernatant, 100 µl DMSO was added to dissolve the formazan. The absorbance was measured at wavelength of 490 nm by using ELISA reader (ASYS Hitech GmbH, Austria).

ELISA assay

To determine an inflammatory response, cells were adjusted to 1 × 10⁵ cells/mL, plated in 24 wells and incubated at 37°C in a humidified incubator with 5% CO₂ and 95% air for 48 h in the presence of 20 µl of ST at the concentrations of 8, 4, 2 and 1 mg/mL, plus 20 µl ConA (50 µg/mL) were added to wells (three wells for each experiment), using as control spleen cells only treated with ConA. Supernatants were then collected and stored at -70°C. IL-2 and IFN-γ of Th1, IL-4, IL-5 and IL-13 of Th2 were then measured in the culture supernatants by ELISA at 450 nm in a microplate reader. All data represent means ± standard deviations of at least three different replicate from three independent experiments.

RT-PCR for detecting mRNA of GATA-3, T-bet and COX-2

Total RNA was extracted from lung tissue under the manufacturer's instructions using trizol reagent. cDNA obtained from 0.5 µg total RNA was used as a template for COX-2, GATA-3 and T-bet PCR amplification. Mouse β-actin was used as an internal standard for each PCR reaction. PCR for all mRNA was carried out with initial denaturation at 94°C for 5 min, followed by 45 cycles of denaturation at 94°C for 1 min, annealing at 56°C, and extension at 72°C for 1 min, and a final extension at 72°C for 10 min. 1.5% (w/v) agarose gels and visualized by ethidium bromide staining was supplied to separate the final products of PCR. The results were photographed and calculated with the aid of soft image J 1.44. The primer sequences used were as follows:

β-Actin

Forward: 5'-TGCTGTCCCTGTATGCCTCT-3'
Reverse: 5'-TTTGATGTCACGCACGATTT-3'

GATA-3

Forward: 5'-AGGGCTACGGTGCAGAGGTA-3'
Reverse: 5'-CGGAGGGTAAACGGACAGAG-3'

RT-PCR for detecting mRNA of GATA-3, T-bet and COX-2

Total RNA was extracted from lung tissue under the manufacturer's instructions using trizol reagent. cDNA obtained from 0.5 µg total RNA was used as a template for COX-2, GATA-3 and T-bet PCR amplification. Mouse β-actin was used as an internal standard for each PCR reaction. PCR for all mRNA was carried out with initial denaturation at 94°C for 5 min, followed by 45 cycles of denaturation at 94°C for 1 min, annealing at 56°C, and extension at 72°C for 1 min, and a final extension at 72°C for 10 min. 1.5% (w/v) agarose gels and visualized by ethidium bromide staining was supplied to separate the final products of PCR. The results were photographed and calculated with the aid of soft image J 1.44. The primer sequences used were as follows:

β-Actin

Forward: 5'-TGCTGTCCCTGTATGCCTCT-3'
Reverse: 5'-TTTGATGTCACGCACGATTT-3'

GATA-3

Forward: 5'-AGGGCTACGGTGCAGAGGTA-3'
Reverse: 5'-CGGAGGGTAAACGGACAGAG-3'
Figure 2. The microstructure of *S. tuberosa*.

T-bet

Forward: 5' –CCCATTCCTGTCCTTCACCG-3'
Reverse: 5' –CATGCTGCCTTCTGCCTTTC-3'

COX-2

Forward: 5' –GAAGTCTTTGGTCTGGTGCCTG-3'
Reverse: 5' –GTCTGCTGGTTTGGAATAGTTGC-3'

Lung tissue histopathology

The lung tissues were fixed in 10% (v/v) neutral buffered formalin for 24 h at 4°C. Then tissues were embedded in paraffin, cut into sections of 4 µm thickness, and stained with H&E solution. Tissues were subsequently mounted under cover slips using DAKOmounting medium (Dakocytomation, Denmark, CA), and inflammation response evaluated.

Statistical analysis

Data represent means ± SD and differences were considered significant at P<0.05. Statistical significance was determined using the Student’s two-tailed t-test to compare independent means, and the two-way analysis of variance (ANOVA) test using the program SPSS 13.0.

RESULTS

Identification of *Stemona* species

Chinese medicinal herb Buibu was purchased from Haozhou, Anhui province. Nine species *Stemona* exist in China and three were included in Pharmacopoeia of the People’s Republic of China; different species showed different antitussive capacity (Hu et al., 2009). It is necessary to identify the species before illustrating the immunoregulatory effects of this herb.

As shown in Figure 2, there were about three root cells, strong wood of the wall, and there were no fine stripes; moreover, most of the cells lined the inner wall thick. Casual fibrous with a square type was outer edge of the cortex, or non-wood wall-wood. Phloem beam were 36 to 40, arranged inter phase, xylem catheter was round polygon, and wood fiber and wood and wood-parenchyma cells connected ring layer; root marrow fiber less was in a single casual. All the character belonged to ST.

Effects of ST on splenocyte proliferation *in vitro*

The antibacterial activities, antitussive functions of ST indicated that ST might have potent immunomodulatory function. To evaluate the effect of ST on the immune system, we evaluated the proliferation of spleen cells from mice in response to ConA. As shown in Figure 3, ConA-induced splenocyte proliferation was significantly attenuated by ST in a concentration-dependent manner.

Effects of ST on T-cell cytokine responses *in vitro*

As shown in Figure 4, the secretion of Th1 and Th2 cytokines was significant increased compared with control group (P<0.005 or P<0.05). Compared with ConA group, ST stimulated IL-5 (P<0.05) at the tested
Concentration and IL-13 (P<0.05) at 0.1 mg/mL. Th1 cytokines was increased (P<0.05 or P<0.005) at 0.8 mg/mL.

The expression of cytokine levels in ST treated mice spleen cells

We then tested cytokine responses in supernatants of spleen cells isolated from ST treated mice. Results are shown in Figure 5. OVA inhalation in sensitized mice caused a significant decrease in IL-2 (P<0.05) and IFN-γ (P<0.005) and a distinctly increase in IL-4 (P<0.05), IL-5 (P<0.05) and IL-13 (P<0.05) compared with PBS control mice. ST treatment significantly increased the levels of Th1 cytokines (P<0.005 or P<0.05) and suppressed the concentrations of Th2 cytokines (P<0.005 or P<0.05), in a dose-dependent manner.
Figure 5. Cytokines production in spleen culture supernatants. Individual splenocytes from each treated group were isolated and cultured in medium containing ConA (5 µg/mL). Supernatants were collected after 72 h of culture. (A), IFN-γ and IL-2; (B), IL-4, IL-5 and IL-13 levels were determined by ELISA. Data are represented as means ± SD from 6 to 10 individual mice in each group. *, P<0.05; **, P<0.005 compare to PBS control.

Effect of ST on lung histology

We evaluated the histologic effects of ST on airway inflammation; lung tissues were collected and fixed with H&E 48 h after the final OVA challenge. Inflammatory cells were present in the peribronchial and perivascular tissues in lung sections of OVA-sensitized/challenged mice compared to PBS mice (Figure 6). Numbers of infiltrating cells were significantly increased in OVA group, however, moderately lowered after ST treated.

Effect of ST on T-bet, GATA-3 and COX-2 mRNA expression

The mRNA expression of T-bet, GATA-3 and COX-2 of OVA induced mice were detected by RT-PCR (Figure 7A). As a key transcription factor to direct Th1
Figure 6. Effects of ST on the inflammation in lung tissue. Histological examination of lung tissue was performed 48 h after the final OVA challenged. Lung tissues were cut into 4 μm thickness, and stained with H&E solution. NC, PBS control; OVA, OVA control; ST-1, 125 mg/kg ST treated; ST-2: ST 250 mg/kg treated.

differentiation, T-bet mRNA expression proved to be strongly inhibited in OVA mice model (Figure 7B), suggesting a defective Th1 commitment status. However, T-bet mRNA expression was evoked distinctly by oral ST. Conversely, the expression of GATA-3, a transcription factor controlling Th2 differentiation, was evidently increased in OVA mice model, the increasing level was reduced by ST interestingly (Figure 7B). GATA-3/T-bet ratio (Figure 7B) in OVA mice was significantly higher than that in PBS mice (P<0.005), and ST decreased the ratio. COX-2 is a key factor for the progression of inflammation, but in our study, ST had no remarkable effect on the COX-2 expression compared to OVA treated mice (Figure 7C).

DISCUSSION

Extracts from roots of the herb Stemona are used to relieve respiratory disorders, and used as an insecticide for enterobiasis and lice in traditional China for thousands of years. Researchers showed that the alkaloids are the main active ingredients (Xu et al., 2010), about 100 stemona alkaloids were isolated from Stemonaceae species (Pilli et al., 2000). This study is the first to provide experimental evidence demonstrating that ST modulates immune responses in vitro and in OVA-induced asthma mice.

Many studies have shown that T-cells play a prominent role in immune responses system. CD4+ Th cells could be functionally divided into Th1 and Th2 groups based on their patterns of cytokine production (Mosmann et al., 1986). The ratio of Th1 to Th2 cells is balanced under normal physiologic conditions. But, when the balance between Th1 and Th2 is broken, diseases caused by Th1 or Th2 polarization will occur. Different patterns of T-cell differentiation result in the activation of different inflammatory effector pathways. For example, Th1 polarization result in atopic dermatitis, asthma, and most tumors diseases (Arakawa et al., 2004; Nakamura and Hoshino, 2005; Horiiuchi et al., 2007), whereas Behcet disease and graft rejection result from Th2 polarization (Li et al., 2003; Obata et al., 2005).

In the present work, we studied the immunoregulatory effect of crude methanolic extract of the Chinese traditional medicine ST in vitro by murine spleen cells. The MTT method was used to detect effect of ST on spleen cells proliferation; results indicated that ST showed remarkable ability to reduce the increased ConA-induced spleen cells proliferation. ST stimulated IL-5 (P<0.05) at the tested concentration and IL-13 (P<0.05) at 0.1 mg/mL, and increased Th1 cytokines (P<0.05 or P<0.005) at 0.8 mg/mL. However, these results indicated that ST could modulate the balance of Th1/Th2 which related to immune system.

Asthma is classified as an inflammatory disease partly caused by imbalance of Th1/Th2. Levels of cytokines in Th1 and Th2 cells were determined by ELISA in OVA...
Figure 7. Analysis of T-bet mRNA, GATA-3 mRNA and COX-2 mRNA in the lung of OVA initiated mice from 6 to 10. The expression of T-bet mRNA and GATA-3 mRNA was evaluated by image J 1.44. The housekeeping gene β-actin from the same samples was amplified as a control. (A), T-bet mRNA, GATA-3 mRNA and COX-2 mRNA levels are presented as means ± SD. NC, PBS treated only; OVA, OVA treated; ST-1, 125 mg/kg ST treated + OVA challenged; ST-2, 250 mg/kg ST treated + OVA challenged; ST-3, 500 mg/kg ST treated + OVA challenged. (B), GATA-3/T-bet ratio are presented as means ± SD. insert: IOD/area of T-bet and GATA-3 mRNA. **, P<0.005 compared with PBS control. ##, P<0.005 compared with OVA control. (C), IOD/area of COX-2 mRNA expression.
challenged and sensitized asthma mouse model. ST decreased levels of IL-4, IL-5 and IL-13 in supernatants of ConA-activated spleen cells cultures, at the same time, increased levels of IL-2 and IFN-γ. Thus, ST showed strongly modulation on the imbalance of Th1/Th2, and it may be a useful medicine for asthmatic patients. T-bet, is reported to induce IFN-γ production while inhibiting IL-4 production. GATA-3, a member of the GATA family of zinc finger proteins, is recognized as a Th2-specific transcription factor and plays an important role in the development of the Th2 phenotype while inhibiting Th1 development. Therefore, it has been reported that changes in the T-bet/GATA-3 ratio reflect changes in the Th1/Th2 balance. So the ratio of GATA-3/T-bet may be used to access the immune balance between the Th2 and Th1 responses in mice. RT-PCR results showed that lung tissues from OVA treated mice expressed low levels of T-bet mRNA and high levels of GATA-3 mRNA compared with PBS control, and that the GATA-3/T-bet ratio was significantly higher than that in PBS control. ST inhibited the ratio in a dose-dependent manner. COX-2 is the rate limiting enzyme and responsible for the catalysis of prostaglandin E2 (PGE2) from arachidonic acid and is a key factor for the progression of inflammation (Surh et al., 2001). Although the inflammation cells around peribronchial and perivascular in OVA-induced mice were inhibited by ST in the H&E detection, ST showed an obviously influence on COX-2 expression in the asthma mice. Together, these findings indicated the pharmaceutical immuno-regulatory effects of ST on the Th1/Th2 balance depended on modulating the T-bet/GATA-3 ratio.

In conclusion, we have demonstrated that the ethanol extracts from ST had significant immuno-regulatory properties as seen in the in vitro and in asthma model induced by injection of OVA. The balance of Th1/Th2 of spleen cells and in the mainly controlling proteins T-bet/GATA-3 of OVA-induced asthma mice lung tissues were distinctly modulated by ST. And ST strongly inhibited inflammation cells in OVA challenged asthma mice. Though further investigation to identify the biologically active ingredients of this extract are being undertaken, ST may be a potential medicine for immune disorder, especially for allergic asthma.

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REFERENCES


