

Modulation of Antigen-Induced Anaphylaxis in Mice by a Traditional Chinese Medicine Formula, Guo Min Kang

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Abstract: A traditional Chinese Medicine (TCM) formula, *Guo Min Kang* (GMK), has been used in clinics in China for allergic diseases, including type I immediate hypersensitivity, a potentially fatal disease, but its modulatory mechanism remains elusive. The aim of this study was to investigate the modulatory mechanisms of GMK in a mouse model of Ag-induced anaphylaxis. Ag (conalbumin) sensitized mice were treated with either PBS (sham) or GMK before (schedule A) or during (schedule B) sensitization, and various anaphylactic parameters were measured following Ag challenge, including symptom score, cutaneous hypersensitivity response, mast cell degranulation, plasma histamine levels and the levels of specific IgE and T-cell responses. Systemic anaphylaxis was investigated in mice immediately following Ag challenge, and the results showed that GMK-treated mice from both treatment schedules A and B showed significantly reduced symptom scores when compared with the sham-treated group. The reduction in symptom score was associated with a significant reduction in the level of Ag-induced cutaneous immediate hypersensitivity. Also, GMK was able to suppress Ag-induced IgE production and T-cell responses, while it spares mitogen (Con A)-induced T-cell response. Further, treatment of mice with GMK abrogated the levels of Ag-induced

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histamine release and significantly reduced the number of degranulated mast cells. No effect of GMK was observed on the levels of total IgE and plasma histamine in naive mice. These results provide a basis for the modulation effect of GMK and suggest a potential utility of GMK as a prophylactic and therapeutic agent.

Keywords: Chinese Herbal Medicine; Guo Min Kang; Ma-Xing-Shi-Gan-Tang; Allergic Diseases; Anaphylaxis; Mouse.

Introduction

Type 1 immediate hypersensitivity or anaphylaxis is one of the most common immune disorders. Immunoglobulin E (IgE) is the primary antibody involved in the initiation of immediate hypersensitivity by triggering the release of histamine and other mediators from the mast cells or the basophils (Hephen and Church, 1993; Martin *et al.*, 1989; Wasserman, 1983a; Ishizaka *et al.*, 1971; Conrad *et al.*, 1975). Current therapy of atopic diseases has included the use of pharmacological agents such as antihistamine and corticosteroids that inhibit the mast cell degranulation and/or the inflammatory effects of mediators; it also involves the use of immunization protocol that stimulates the production of “blocking antibodies” of the IgG class and, in some instances, inhibits IgE secretion (Patterson *et al.*, 1983; Lichtenstein and Fauci, 1986; Terr, 1994). However, the limited efficacy and potential side-effects of current therapies demand the development of newer and more effective therapies. Indeed, several newer approaches have been under extensive evaluation (for review see Finegold, 2004). For example, the use of humanized anti-IgE Abs has shown promise, but is associated with a relatively high cost.

Because of its effectiveness, low cost and relative absence of side-effects there is increasing interest in the use of Traditional Chinese Medicine (TCM) for the treatment of various diseases including allergic disorders (Li and Srivastava, 2006; Li, 2007; Huang *et al.*, 2008; Jordan and Tu, 2008). For example, Kampo medicine originally derived from TCM is widely used in Japan. Studies utilizing TCM formulae, such as “*xiao qing long tang*” and “*xiao chai hu tang*”, which are mostly derived from the classic medical works, have shown clinical efficacy in the treatment of atopic rhinitis and asthma (Matsumoto *et al.*, 1993; Homma *et al.*, 1993; Masyo *et al.*, 1994; Kumagai, 1988). A clinical trial study has demonstrated the effectiveness of TCM for the treatment of atopic eczema (Sheehan *et al.*, 1992; Latchman *et al.*, 1994), which is associated with a decrease in the level of serum IgE complex (Latchman *et al.*, 1995; Latchman *et al.*, 1996). Numerous experimental studies have demonstrated pharmacological effects of purified active ingredients derived from plants used in TCM and Kimpo formulae. Glycyrrhizin has been shown to modulate IgE and mast cell degranulation (Imanishi *et al.*, 1989) and to exhibit anti-inflammatory effects (Schleimer, 1991; Tamaya *et al.*, 1986). Recent clinical studies suggested that several herbal remedies are effective in treating mild-to-moderate persistent asthma (Wen *et al.*, 2005; Hsu *et al.*, 2005; Chan *et al.*, 2006; Chang *et al.*, 2006a). The potential utilities of TCM formulas have also been provided by murine models of asthma and type I hypersensitivity (Li *et al.*, 2000; Srivastava *et al.*, 2005; Chang *et al.*, 2006b; Xiong *et al.*, 2007; Cho *et al.*, 2008).

Traditionally, the TCM formula “Ma-Xing-Shi-Gan-Tang” was prescribed to treat allergic rhinitis and asthma. In the clinical practice in China, we have successfully used *Guo Min Kang* (GMK), a modified prescription of “Ma-Xing-Shi-Gan-Tang” for treating patients with allergic rhinitis, allergic asthma, urticaria and other disorders. However, the regulatory mechanisms of GMK remain to be defined. To this end, we have used an IgE-dependent, Ag-induced anaphylaxis mouse model, including several immunological parameters, to investigate the modulatory mechanisms of GMK on Type 1 immediate hypersensitivity.

Materials and Methods

Mice, Immunization and Challenge

Male AKR/J mice, 6–8 weeks of age, were purchased from Jackson Laboratory (Bar Harbor, ME) and maintained under specific pathogen-free conditions. Mice were sensitized intraperitoneally with 500 μ g of conalbumin (CA), a model antigen, adsorbed in 2 mg of aluminum hydroxide in 0.4 ml of PBS on day 0. Sham-immunized mice received an equal amount of PBS. On day 21 post immunization, mice were challenged intraperitoneally with 500 μ g of CA in 0.4 ml of PBS.

GMK Preparation and Treatment Protocol

GMK, the medicine tested in the experiments, was prepared as spray-dried powder of extracts from 12 Chinese medicines, and manufactured at Gao You Pharmaceutical Factory (Jiang Su, China) in capsule form (0.3 g/capsule). The 12 components of GMK are: Ma-Huang (Herba Ephedrae), Xing-Ren (Semen Armeniacae Amarum), Shi-gao (Gypsum Fibrosum), Gang-Cao (Radix Glycyrrhizae), Ku-Shen (Radix Sophorae Flavescentis), Jiang-chan (Bombyx Batryticatus), Chan-yi (Cryptotympana pustulata Fabricius), Zi-cai (Lithospermum erythrorhizon), Sheng-shan-zha (Crataegus pinnatifida Bge.), Zhi-qiao (Citrus aurantium L.), Lu-lu-tong (Fructus liquidambaris), Nu-zhen-zi (Fructus Ligustri Lucidi). The powder was dissolved in water to the concentration of 50 mg/ml. Mice were treated daily with GMK (1 g/kg) from Day 7 to Day 21 (starting 7 days before initial immunization, Schedule A), or from Day 7 to Day 21 (7 days after the initial Ag sensitization; schedule B). The dose of GMK used in this study was chosen based on an equal effective dose between human and animal by calculating the ratio of the body surface areas (Xiu *et al.*, 1986). The solution containing GMK was administered intragastrically once a day. The control groups included sham- and Ag-sensitized mice receiving an equal volume of water as sham treatment. A group of naive mice receiving equal dosage of GMK was also included as additional control.

Systemic Anaphylaxis Testing

Symptoms of anaphylaxis became apparent in mice at 5 to 10 min, and peaked at 20 to 40 min, after Ag challenge. The severity of symptoms was determined by observing mouse behavior post challenge, and was judged by their responses to stimuli such as prodding. Shock was

scored by the following scoring system — 0, no sign of shock; 1, scratching and rubbing around the nose and head; 2, less activity or standing still with an increasing respiratory rate or puffing around the eyes; 3, asthmoid respiration, cyanosis around the mouth and the tail; 4, slight or no activity after prodding or tremor and convulsion; 5, death. This scoring system was established based on our previous experiments and a modified scoring system used previously (Srivastava *et al.*, 2005; Roy *et al.*, 1999; McCaskill *et al.*, 1984).

Intradermal Skin Testing

Mice were tested for immediate cutaneous hypersensitivity by intradermal skin test on Day 21 at the time of challenge as previously described with a slight modification (Brocklehurst, 1978; Saloga *et al.*, 1993). Briefly, the skin of the belly was carefully shaved during anesthesia induced by intraperitoneal injection of a mixture of ketamine and xylazine (80 mg/kg and 10 mg/kg, respectively). For each skin test, 20 μ l of CA solution (1.25 mg/ml) was injected intradermally with a 30-gauge needle while the skin was stretched taut. This antigen concentration was found to produce consistent wheal reactions by previous titration experiment. The wheal reactions were assessed after 20–30 min. A reaction was scored as positive if the wheal diameter was > 3 mm in any direction, measured with a transparent ruler (skin test ruler). Evaluation of wheal formation was always carried out in a blinded fashion.

Determination of Serum CA-Specific IgE Level

The blood was obtained through orbital plexus puncture once a week for 3 weeks from each group of mice. The levels of CA-specific serum IgE were measured by ELISA. Immulon II round-bottom plates (Dynatech Laboratories, Inc., Chantilly, VA) were coated with 25 μ g/ml CA in PBS. After overnight incubation at 4°C, plates were washed 3 times with PBS/0.05%-tween 20 and blocked with 1% BSA-PBS for 1 hour at 37°C. After 3-time washings, serum samples were diluted 5-fold in 1% BSA-PBS, and incubated overnight at 4°C. Plates were then washed and 100 μ l of goat anti-mouse IgE (0.3 μ g/ml; Sigma, St. Louis, MO) Ab was added to the wells for an additional 2 hours at 37°C. After 3x washings, 100 μ l of donkey anti-goat IgG Ab conjugated with peroxidase (0.3 μ g/ml) was added for an additional 1 hour at 37°C. The reaction was developed with TMB (Bio-Rad Laboratories, Hercules, CA) 30 min at RT and stopped with the addition of 1 N H₂SO₃, and read at 450 nm. The level of IgE was calculated by comparison with a reference curve generated by using a mouse MAb, anti-DNP IgE (Sigma, St. Louis, MO). Briefly, DNP-conjugated bovine serum albumin (DNP-BSA) was coated at the same concentration as CA. After overnight incubation at 4°C, the plates were washed and blocked the same way as described above. Ten serial 1:2 dilutions of mouse anti-DNP IgE starting from 1000 ng/ml were added.

Antigen- and Mitogen-Induced T-Cell Proliferative Responses

The spleen was removed aseptically from each group of 6–8 mice 21 days after sensitization, and the single-cell suspension was made in RPMI medium supplemented with

10% FCS, 100 U/ml of penicillin, 100 μ g/ml of streptomycin. 10×10^5 of spleen cells in 200 μ l/well were cultured in the presence or absence of mitogen (Con A), or antigen (CA) at 37°C in a humidified CO₂ incubator for 4 days, and then pulsed with 1 μ Ci per well of ³H-thymidine for the final 18 hours of culture. The cells were harvested and the incorporated radioactivity was counted on a beta-scintillation counter. The results were expressed as counts per min (CPM) or stimulation index (SI; CPM of Ag- or mitogen-stimulated culture/CPM of culture with medium alone).

Determination the Levels of Plasma Histamine

The blood was collected from each mouse 5 to 8 min post challenge. 0.3–0.5 ml of blood was collected into the chilled tube containing 30–40 μ l of 7.5% potassium-EDTA, then chilled immediately on ice and centrifuged (1,500 rpm) for 10 min at 4°C. The plasma was collected and frozen at –80°C until used. The levels of histamine were determined by using enzyme immunoassay kit (ImmunoTECH Inc., ME). Briefly, 100 μ l of acylated standards or 1:100 diluted acylated plasma samples were added to the microtiter plates precoated with anti-histamine MABs, and then 200 μ l of histamine acetylcholinesterase conjugate was added to each well. After incubation at 4°C for 18 hours and 3 $\times$ washings, 200 μ l of substrate was then added and incubated at RT for 20 min. The reaction was stopped by the addition of 50 μ l of stop solution. The plate was read at 410 nm. The titers of histamine were calculated by comparison with the standards provided by the manufacturer.

Histologic Studies

For analysis of mast cell degranulation, the ear samples were collected immediately after anaphylaxis-related death or 40 min after challenge for mice that survived. The tissues were fixed in 4% paraformaldehyde, 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.3, at room temperature for 30 min, then washed and stored in the same buffer at 4°C until processing into 3 μ m paraffin-embedded, toluidine blue-stained sections. A degranulated mast cell was defined as a toluidine-positive cell with 5 or more distinct stained granules completely outside of the cell (Snider *et al.*, 1994). One histology section from each of the 3 sites of each mouse ear was examined under the light microscope (400 $\times$) by an observer unaware of their identities. Four hundred mast cells were classified for each ear sample. The organs (spleen, kidney, heart, and lung) from GMK-treated naive mice and the untreated naive mice were obtained, and fixed in 10% formalin. The tissue sections were examined as described above.

Results

Effect of GMK on Ag-Induced Systemic Anaphylaxis and Immediate Cutaneous Hypersensitivity

AKR/J mice were treated daily with 1 g/kg of GMK from Day 7 (schedule A) or Day 7 (schedule B) until Day 21 after immunization. Systemic anaphylaxis was investigated

Table 1. Effect of GMK on Ag-Induced Immediate Cutaneous Hypersensitivity

Sensitization/Treatment	Challenge	Number	Positive Responder %
PBS	CA	0/10	0
CA	CA	9/11	82
GMK/CA (A)	CA	2/10	20*
GMK/CA (B)	CA	3/10	30**

The numbers and percentages of positive responders were derived from 2 different experiments. Statistical analysis was performed by using Wilcoxon signed-rank test.

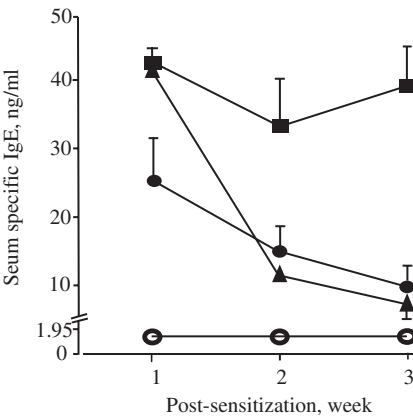


Figure 2. Inhibitory effect of GMK on the level of CA-specific IgE. Sera from different groups of mice as indicated: PBS (open circle), n = 5; CA (square), n = 4; GMK (schedule A)/CA (solid circle), n = 7; CA/GMK (schedule B) (triangle), n = 5 were obtained 3 hours before challenge. The level (ng/ml) of CA-specific IgE was measured by ELISA as described in Materials and Methods.

sham-treated mice at Day 7 through Day 14 (33.5 ng/ml), and at Day 21 (39.79 ng/ml). In contrast, the level of specific IgE in GMK (schedule A)-treated mice was significantly decreased at Day 7 (30.812 ng/ml), and also at Day 14 (15.188 ng/ml) and Day 21 (10.064 ng/ml). Interestingly, the level of specific IgE in GMK (schedule B)-treated mice was significantly decreased (12 ng/ml) at Day 14 after one week treatment with GMK, and the level was similar to that of sham-sensitized mice (7.96 ng/ml) at Day 21, 2 weeks after treatment. The total IgE level in naive mice after treatment with GMK was the same as that seen in untreated naive mice (data not shown).

Effect of GMK on Ag or Mitogen-Induced Lymphocyte Stimulation

To determine the effect of GMK on the T-cell responses, spleen cells were prepared from different groups of mice 21 Days after sensitization, and cultured with or without Ag or mitogen as a positive control. As shown in Fig. 3, while the expected significant increase in

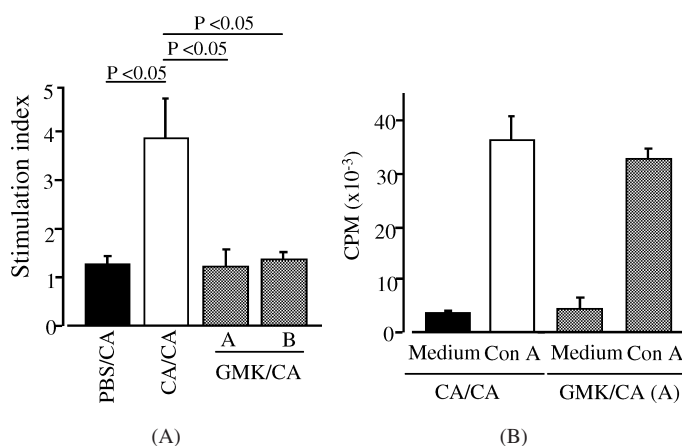


Figure 3. Proliferative response of spleen cells to Ag (CA) or a mitogen (Con A), which is expressed either as “stimulation index” or counts per min (CPM). Statistical analysis was performed by using t-test. Note that there is no statistical difference for the proliferation response to a mitogen (Con A) between spleen cells from GMK (schedule A)-treated mice and sham-treated mice (panel B).

Ag-induced proliferative response was found in T cells from CA-sensitized and sham-treated mice (CA/CA) when compared with those seen in the sham-sensitized group (PBS/CA), the Ag-induced T-cell proliferative response was significantly reduced in CA-sensitized mice that were treated with GMK of both schedules A and B (GMK/CA). These results suggest that GMK inhibits the induction of Ag-specific T cells *in vivo*. Moreover, at the treatment dosage, spleen cells from GMK (schedule A)-treated mice showed similar levels of proliferation to a mitogen, Con A (Fig. 3B), suggesting that the suppression of lymphocyte proliferation was Ag specific.

Effect of GMK on the Level of Plasma Histamine and Mast Cell Degranulation

To further determine whether the effect of GMK on type 1 immediate hypersensitivity could be attributed, at least in part, to the reduction of mast cell histamine release, we first examined the plasma histamine levels. As shown in Fig. 4A, the level of plasma histamine in the CA-sensitized group (202 ng/ml) was significantly increased after Ag challenge when compared with the sham-sensitized group (40.2 ng/ml). Consistent with the decrease in the level of serum Ag-specific IgE, the elevated histamine level was significantly decreased in GMK (schedule A)-treated (61.43 ng/ml) and in GMK (schedule B)-treated (69.66 ng/ml) groups. Similar to the level of total IgE, the level of plasma histamine in naive mice after treatment with GMK was the same as that seen in untreated naive mice.

These findings were further corroborated by histologic examination of mast cell degranulation of the ear samples from mice after CA challenge. The results are shown in Fig. 4B. While the number of degranulated mast cells was markedly increased in the CA-sensitized group when compared with the sham-sensitized group, a significant decrease of the percentage of degranulated mast cells was found in GMK (schedule A)-treated as well as in GMK

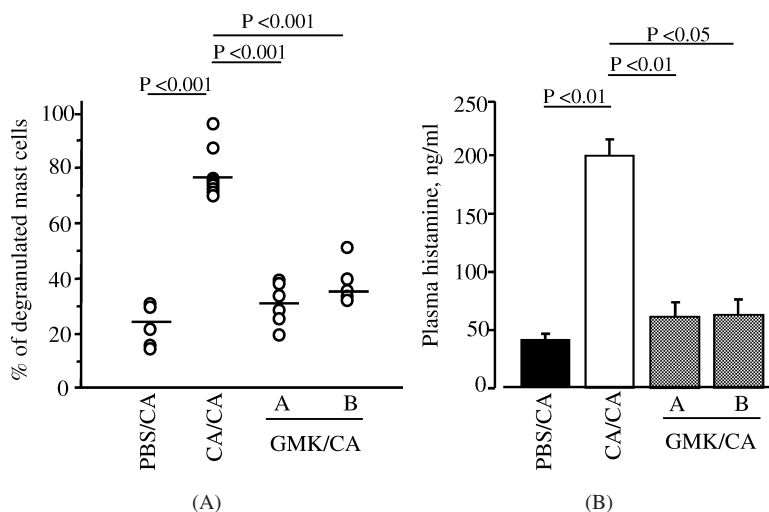


Figure 4. (A) Effect of GMK on mast cell degranulation (expressed as percentage of degranulated mast cells out of 400 mast cells analyzed). Each symbol represents each individual mouse. Statistical analysis was performed by using t-test. (B) Inhibitory effect of GMK on the level of plasma histamine. Plasma from different groups of mice as indicated: PBS, $n = 5$; CA, $n = 5$; GMK (schedule A)/CA, $n = 7$; CA/GMK (schedule B), $n = 5$ were obtained 5–8 min after challenge. Statistical analysis was performed by using t-test.

(schedule B)-treated groups (31.96% and 33.96%, respectively) when compared with the sham-treated group (78.8%). The differences between GMK-treated groups and the sham-sensitized group are not statistically significant. In addition to the findings that no effect of GMK was observed on the levels of total IgE and plasma histamine in naive mice, the tissues including spleen, thymus, kidney, and lung from naive mice after treatment with GMK showed no apparent pathological changes (data not shown).

Discussion

According to TCM theory, “Ma-Xing-Shi-Gan-Tang” can redirect flow of lung qi, calm wheezing, and clear heat. Traditionally, this formula has been used to treat wheezing, cough, and difficulty in breathing. To achieve better efficacy for treating allergic diseases such as asthma, allergic rhinitis and urticaria, we modified this prescription by adding several components (see “Materials and Methods”), based on TCM principles, our clinical experiences, as well as modern pharmacological studies of individual herbal extracts. This modified traditional prescription, GMK, has been used clinically to treat patients with allergic rhinitis, allergic asthma, and urticaria in China. In this study, we demonstrated that GMK has an inhibitory effect on Ag-induced type 1 immediate hypersensitivity in mice, and that its efficacy is associated with reduction of Ag-specific IgE, Ag-induced T-cell proliferation and mast cell histamine release. Also of significance is the finding that GMK does not alter the levels of either total serum IgE or the basal plasma histamine in mice. These results provide the first experimental basis for the clinical efficacy of GMK.

Type 1 immediate hypersensitivity, such as systemic and cutaneous anaphylactic reactions, is provoked by histamine, serotonin and some other mediators released from mast cells and basophils (Ishizaka and Ishizaka, 1975). IgE is clearly essential to the pathogenesis of the type 1 allergic responses (Ishizaka *et al.*, 1974). In this study, the decreases in the levels of serum Ag-specific IgE and plasma histamine after treatment with GMK may be the main pharmacological action of GMK on immunoregulation of allergic diseases. The reduction of IgE and histamine levels were observed not only before immunization (schedule A) but was also found after immunization (schedule B), suggesting that GMK is effective in regulating both the primary and secondary immune responses.

The decreased vascular permeability may result from the reduction of histamine because histamine is a major vasoactive mediator released by activated mast cells (Oettgen *et al.*, 1994; Wasserman, 1983b). Histological analysis of ear mast cells revealed that GMK reduced mast cell degranulation, suggesting that the inhibitory effect of GMK on the elevated plasma histamine is at least partially due to the decreased mast cell degranulation. This inhibitory effect of GMK on mast cell degranulation may result from the decrease of Ag-specific IgE following the treatment of mice with GMK. Another possibility is that GMK may directly act on the mast cell membrane to stabilize the cell membrane and therefore prevent the mast cell degranulation. Desensitization of mast cells may be also due to reduction of antigen specific IgE. Further study is needed to examine the molecular mechanism of GMK on the inhibition of mast cell degranulation. GMK has no effect on the levels of total IgE and histamine in naive mice receiving the same doses of GMK *in vivo*. These findings suggest that GMK acts, selectively, on the allergic state but not on the normal immune system. This was further confirmed by the histologic analysis which showed that the organs such as spleen, thymus and kidney appeared to be normal in naive mice after treatment with GMK.

GMK is a mixture of 12 Chinese medicines. Four of which, Herba Ephedrae, Semen Armeniacae Amarum, Gypsum Fibrosum and Radix Glycyrrhizae, are widely used for the treatment of asthma. A recent study found that *shao qin long tong* inhibits histamine release from mast cells *in vitro* (Matsumoto, 1993). *Ma xing shi gan tong* has an inhibitory effect on type 1 hypersensitivity (Li, 1983) and antigen induced immediate asthmatic responses (Kao *et al.*, 2001). Four components of GMK (Herba Ephedrae, Semen Armeniacae Amarum, Citrus aurantium L. and Radix Sophorae Flavescentis) are components of several TCM formulas which have been shown to be effective in the treatment of asthma (Chan *et al.*, 2006; Li *et al.*, 2000; Cho *et al.*, 2008). However, the effects of some other medicines in GMK on immunologic changes have not been studied. Regarding the active components, previous studies have shown that glycyrrhetic acid, a purified extract from Radix Glycyrrhizae effectively inhibited dexamethasone-stimulated histamine synthesis from mastocytoma P-815 cells and histamine release from antigen-stimulated sensitized mast cells (Sheehan *et al.*, 1992). However, the exact active components in this mixture are at present not known. Because type 1 immediate disorder is a multifactoral allergic disease involved in both humoral and cellular abnormalities, this led us to speculate that GMK probably possesses many actions, and that the efficacy of GMK on the type 1 immediate hypersensitivity may work through its multi-targets and may regulate various immunological responses. A previous study of the classic formula TCM TJ-19 (*xiao qin long tang*) showed that the whole formula was more effective

in the inhibition of IgE-mediated PCA than each single component (Masys *et al.*, 1994). In addition to the therapeutic potential of GMK, further studies are needed on the identification of active components and sequential events leading to the immunologic changes.

In conclusion, GMK possess both prophylactic and therapeutic effects on Ag-induced type 1 immediate hypersensitivity in this mouse model. This effect is associated with down-regulation of the specific IgE levels, T cell proliferative responses and mast cell histamine release. In addition, GMK showed no apparent adverse effect on the normal immune response in this model. Although GMK is widely used in China, this study provides the first experimental basis for its pharmacological actions in the regulation of allergic responses.

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