Suppressive Effects of Hocho-ekki-to, a Traditional Chinese Medicine, on IgE Production and Histamine Release in Mice Immunized with Ovalbumin

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We examined the effects of Bu-Zhong-Yi-Qi-Tang (Japanese name: Hocho-ekki-to, HET), a traditional Chinese medicine, on IgE production and histamine release in mice immunized intraperitoneally with a mixture of ovalbumin (OA) and aluminum hydroxide (alum adjuvant). Three groups of mice were orally administered 0, 1.7 or 17 mg of HET on day 13 after the first immunization with a mixture of 1 μg OA and 1 mg alum adjuvant. They were again immunized with the same dose of OA plus alum adjuvant on day 14. The immunological changes in mice treated with OA alone or OA plus HET were examined, and the following findings were obtained. In the HET-treated mice, the elevation of anti-OA IgE in serum, and histamine release from basophils in blood, were significantly suppressed. A significant suppression of interleukin-4 (IL-4) secretion and proliferation of splenic lymphocytes in primary culture was also observed. A tendency to suppress the elevation of anti-OA IgG1 in serum and interleukin-2 (IL-2) secretion from splenic lymphocytes was observed in the HET-treated mice. These findings suggest that oral administration of HET suppresses IgE antibody production and histamine release in type I allergic reaction in mice immunized with OA plus alum adjuvant; this shows the efficacy of HET in treating type I allergic diseases, such as asthma.

Key words traditional Chinese medicine; Hocho-ekki-to; IgE production; histamine release; interleukin-4 (IL-4) production; cell proliferation

There have been many reports describing the biological actions of Kampo-Hozai (traditional Chinese medicines) on immune responses. Xiao-Chai-Hu-Tang (Japanese name; Syo-saiko-to), Xiao-Quing-Long-Tang (Syo-seiryu-to) and Chai-Pu-Tang (Saikoku-to) are well known Kampo-Hozai, and are frequently prescribed for the treatment of asthma in Japan.1–4 Occasionally, Bu-Zhong-Yi-Qi-Tang (Hocho-ekki-to, HET) has also been used in the treatment of asthma.5 It has been reported that oral administration of Syo-saiko-to, Syo-seiryu-to or Saikoku-to suppresses IgE antibody production and histamine release that accompany asthma.6–10 However, there are no reports that confirm the suppressive effects of HET. It has been shown that HET possesses various immunomodulating or immunopharmacological activities in experimental studies: activation of macrophages,11 effects on natural killer cell activity,12–14 interferon inducing activity,15 anti-tumor effects,16 and protection against bacterial infection.17

In the present study, we demonstrate that the oral administration of HET can suppress IgE antibody production and histamine release from basophils in the blood of mice immunized with antigen and adjuvant.

MATERIALS AND METHODS

Animals Eight-week-old female BDF1 mice and male Wistar rats weighing 200 to 300 g were obtained from Charles River Japan Inc. (Atsugi, Japan). They were housed in plastic cages and fed a standard chow diet and water ad libitum.

Materials Ovalbumin (OA; chicken egg, grade VII), RPMI 1640 (without phenol red) and ethidium bromide were purchased from Sigma Chemical Co. (MO, U.S.A.). Alumininum hydroxide (alum adjuvant) was obtained from LSL Co. (Tokyo, Japan). Evans blue was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Crude drugs used to prepare HET were purchased from Uchida Wakanyaku Co. (Tokyo, Japan). Hanks' balanced salt solution (HBSS), fetal calf serum (FCS) and penicillin-streptomycin solution were obtained from Gibco Laboratories, Life Technologies Inc. (NY, U.S.A.). Lympholyte-M was purchased from Cedarlane Laboratories, Ltd. (Ontario, Canada). Flat-bottomed 24- or 96-well plates were obtained from Sumitomo Bakelite Ltd. (Tokyo, Japan). Concanavalin A (Con A) from Oxford GlycoSystems, Inc. (NY, U.S.A.) and lipopolysaccharide (LPS) from Paezel GmbH & Co. (Hanau, Germany) were used. The ELISA kits for mouse interleukin-4 (IL-4) and interleukin-2 (IL-2) were purchased from Endogene Inc. (MA, U.S.A.) and Bender MedSystems (Vienna, Austria), respectively. Cell proliferation ELISA systems kit (version 2), ECL random prime labelling and detection system and Hybond N+ (Nylon membrane) were purchased from Amersham Life Science (Little Chalfont Buckinghamshire, UK). Histamine radioimmunoassay kit was the product of Immunootech. S. A. (Marseille, France). Ultra Spec™ RNA was obtained from Biotex Laboratories (Houston, TX, U.S.A.), RiboGreen™ RNA Quantitation Kit from Molecular Probes (Eugene, OR, U.S.A.), Titan™ One Tube RT-PCR System from Boehringer Mannheim GmbH (Germany) and reverse transcriptasepolymerase chain reaction (RT-PCR) amprimer sets for mouse IL-4, 5′-CCAGCTAGTTGCTCATCCTTCTTTCTCTCTTCG-3′ and 5′-CATGATGTGGAAGTGCCATTCTGACTGAACT-GTGGGCGCTAGAGACCA-3′ and 5′-CTCATTTGATGTGACGACGATTTC-3′, from Clontech Laboratories (CA, U.S.A.).

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Table 1. Composition of HET

<table>
<thead>
<tr>
<th>Crude drug (g/d/ adult human)</th>
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<tr>
<td>Astragalus root</td>
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<tr>
<td>Atractylodes Rhizome</td>
</tr>
<tr>
<td>Ginseng</td>
</tr>
<tr>
<td>Japanese Angelica root</td>
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<tr>
<td>Bupleurum root</td>
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Preparation of HET As shown in Table 1, HET consists of ten kinds of crude drugs and a daily dose of each drug contained in HET for an adult human is prescribed. A mixture of these drugs in 5 fold-distilled water was refluxed for 80 min and the filtrate was lyophilized. The yield was 5.0 g (recovery: 22.2%). Oral intake of the HET extract for an adult human of 60-kg body weight is calculated as 83.3 mg/d/kg body weight. HET was dissolved in distilled water before use.

Immunization and Administration of HET Each mouse, weighing about 20 g, was intraperitoneally immunized with a mixture of 1 μg OA and 1 mg alum adjuvant dissolved in physiological saline solution twice at two-week intervals. Twenty-four mice were divided into three groups of 8 mice each and were orally administered 0, 1.7 or 17 mg of HET on day 13 after the first immunization. The amount of 1.7 mg for mice almost corresponded to the daily dose prescribed for humans. Each mouse was bled from the retroorbital plexus at 2-week intervals after the first immunization. Sera were separated and stored at −80 °C until use.

Measurement of IgE and IgG1 Antibodies The titers of IgE antibody were measured by passive cutaneous anaphylaxis (PCA) assay. Each 0.1 ml aliquot of serial twofold dilutions of serum was injected intraeutaneously into the depilated dorsum of Wistar rats. After 48 h, the rats were challenged intravenously with 1.0 ml of the antigen mixtures in physiological saline solution containing 1 mg OA and 1% Evans blue. The PCA titer was expressed as the reciprocal of the highest serum dilution giving a blue spot of 5 mm or more in diameter, indicating a positive response. IgG1 antibody to OA in serum samples was determined according to the enzyme-linked immunosorbent assay (ELISA) method reported previously. Each well was coated with 5 μg OA, and mouse serum and anti-mouse IgG1 monoclonal antibody were diluted 1:400 and 1:1000, respectively, with 0.01 M phosphate-buffered saline (PBS, pH 7.2) containing 0.05% Tween80 and 1% bovine serum albumin.

Preparation of Splenic Lymphocytes Mice treated with OA alone or OA plus HET (17 mg) were sacrificed under anesthesia with ether on day 28 after the first immunization. The spleens were mashed through stainless mesh with a #75 and rubber spatula. After washing 3 times with HBSS, lymphocytes were isolated from the splenic cells by centrifugation at 500×g for 10 min at room temperature with LympholyteA-M. The cells were washed 3 times with cold HBSS by centrifugation. Viability of the cells was more than 95% as examined by the Trypan Blue exclusion method.

Measurement of IL-4 and IL-2 Splenic lymphocytes, 1×10^6 cells/ml, suspended in 1.0 ml RPMI 1640 containing 5% FCS, 0.2% NaHCO3, and antibiotics containing 100 unit/ml penicillin and 100 μg/ml streptomycin (medium A), were dispensed into a flat bottomed 24-well plate. OA or Con A dissolved in 5 μl RPMI 1640 were added into each well at a final concentration of 10 and 5 μg/ml, respectively. The cells were then cultured at 37 °C in 5% CO2 for 4 d. Culture media were centrifuged at 8000×g for 5 min at 4 °C. The supernatants were stored at −80 °C until use. The contents of IL-4 and IL-2 in the supernatants were measured with ELISA kits.

Proliferation Assay of Splenic Lymphocytes Cells at 2×10^6/200 μl medium A/well, were plated in a flat bottomed 96-well plate. The cells were exposed to 2 μg of OA, 1 μg of Con A or 2 μg of LPS in each well, and cultured at 37 °C in 5% CO2 for 2 d. Cell proliferation was examined by measurement of 5-bromo-2’deoxy-uridine (BrdU) incorporation with the cell proliferation ELISA systems kit in triplicate. The stimulation index of cell proliferation was calculated by the following formula:

\[
\text{index (\%)} = \frac{\text{stimulated cell number} - \text{non-stimulated cell number}}{\text{non-stimulated cell number}} \times 100
\]

Detection of Histamine Release from Basophils Heparinized blood was obtained from the abdominal artery of each mouse on day 28. For the determination of total histamine concentration in blood, 50 μl of the blood was mixed with 1.0 ml of water, frozen, and subsequently thawed. For the detection of histamine released by stimulation of antigen, the blood was diluted 4-fold with 10 mm Tris-HCl buffer (pH 8.0) and 100 μl of the diluted blood was exposed to OA (0.1 ng/ml) for 45 min at 37 °C, and then cooled on ice. The supernatants of both mixtures were obtained by centrifugation at 4000×g for 5 min at 4 °C, and stored at −80 °C until use. The concentration of histamine released from basophils in blood was measured by histamine radioimmunoassay kit. The ratio of the histamine released from basophils to the total histamine in blood was calculated by the following formula:

\[
\text{ratio (\%)} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{total histamine}} \times 100
\]

Gene Expression of IL-4 on Spleen Cells The RNA was prepared from fresh spleen or splenic lymphocytes of the mice on day 28 with Ultra Spec RNA. RT-PCR was carried out according to the one step method using a Titan™ One tube RT-PCR System. 300 ng of RNA and 0.4 μm primer sets of IL-4 or β-actin were added to the reaction mixture (25 μl) with reverse transcriptase and DNA polymerase. The reaction was carried out as follows; reverse transcription at 50 °C for 30 min, denature at 94 °C for 2 min, 25 cycles of amplification (94 °C for 1 min, 63 °C for 1 min and 68 °C for 2 min) and extension at 68 °C for 7 min were continuously performed. After electrophoresis of RT-PCR products on 2% agarose gel, the gel was stained with ethidium bromide. For amplification of signals, DNA was transferred to Hybond N+ and RT-PCR products were confirmed by ECL system. Labeled DNA probes of IL-4 and β-actin were prepared by PCR reaction of pure counterpart derived from cDNA and DNA polymerase reaction with Klenow fragment with fluorescence-labeled deoxyuridine triphosphate (dUTP).

Statistical Analysis Results were given as the mean±S.D. Average values of PCA titers for IgE and antibody responses of IgG were assessed with Bonferroni’s multiple comparison test after Kruskal–Wallis test to examine the dif-
difference between the three groups. Average values of IL-4, IL-2, cell proliferation and histamine release were assessed with the Student's t-test after the F-test to examine the difference between the two groups.

RESULTS

**Effects of HET on IgE and IgG1 Antibody Production**
IgE and IgG1 antibodies were measured by PCA assay and the ELISA method, respectively. The elevation of anti-OA IgG1 antibody production in mice treated with OA plus HET (1.7 or 17 mg) was significantly suppressed compared to those with OA alone (Fig. 1A). Similarly, the elevation of anti-OA IgG1 antibody production was suppressed in mice treated with OA plus HET ($p<0.10$, Fig. 1B).

**Effects of HET on IL-4 and IL-2 Secretion from Splenic Lymphocytes**
Splenic lymphocytes were stimulated with OA or Con A in vitro. In mice treated with OA plus HET, the IL-4 secretions induced by stimulation with OA or Con A were significantly decreased compared with those with OA alone (Fig. 2). A tendency to suppress IL-2 secretion was observed in HET-treated mice ($p<0.10$, Fig. 3).

**Suppression of Proliferation of Splenic Lymphocytes by HET**
Splenic lymphocytes were stimulated with OA, Con A or LPS in vitro. The splenic lymphocytes from mice treated with OA plus HET had a significant reduction in OA response, to 82% of that of mice treated with OA alone (Fig. 4A). The ability of splenic lymphocytes to undergo blastogenesis in response to culturing with Con A or LPS was also investigated. The proliferation of splenic lymphocytes by stimulation with Con A, a T-cell mitogen, was significantly suppressed to 69% of that of mice treated with OA alone (Fig. 4B), but the response of these cells to LPS, a B-cell mitogen, remained unchanged (Fig. 4C). The stimulating index by simulation with OA, Con A or LPS was 268±40%, 510±81% and 345±44%, respectively.

**Suppression of Histamine Release from Basophils by HET**
Blood was exposed to OA in vitro. Histamine release from the basophils in the blood of mice treated with OA plus HET was significantly suppressed compared to those treated with OA alone (Fig. 5). The total histamine contents in the blood of mice treated with OA alone and OA plus HET were 32.8±4.8 and 23.9±5.4 ng/ml, respectively. In both groups,
The spleen lymphocytes of mice treated with OA alone or OA plus HET (17 mg) were cultured in vitro and further stimulated by 2-d incubation with 10 μg OA/ml (A), 5 μg Con A/ml (B) or 10 μg LPS/ml (C). The cell numbers were expressed as the percentage of those obtained by stimulation with OA, Con A and LPS, which gave numbers (100%), 6.4×10⁶, 12.2×10⁶ and 8.3×10⁶, respectively. Values represent the mean±S.D. of 8 mice.

Fig. 5. The Suppressive Effect of HET on Histamine Release from Basophils

The basophils in the blood of mice treated with OA alone or OA plus HET (17 mg) were stimulated with 0.1 ng OA/ml for 45 min, then the histamine concentration in culture media was measured. Values represent the mean±S.D. of 8 mice.

Effects of HET on IL-4 Gene Expression

The gene expression of IL-4 in spleen of mice treated with OA or OA plus HET was investigated. The gel electrophoretic patterns of the RT-PCR products of IL-4 and β-actin are presented in Fig. 6. The expression level of IL-4 mRNA in fresh spleen of mice treated with OA plus HET was lower than that with OA alone, though the expression of β-actin mRNA, a housekeeping gene, was almost constant in each sample, when stained with either ethidium bromide or the ECL system (Fig. 6A). A similar pattern was observed in the RT-PCR products obtained from prepared spleen lymphocytes (Fig 6B).

DISCUSSION

Oral administration of HET significantly suppressed the elevation of IgE antibody in serum, histamine release from basophils, and IL-4 secretion and cell proliferation of splenic lymphocytes compared to those of mice treated with OA alone. Furthermore, it suppressed elevation of IgG1 antibody in serum, and IL-2 secretion and IL-4 gene expression of splenic lymphocytes.

IgE antibody synthesis is induced by IL-4 secreted from helper T cells (Th), especially Th2 type cells. Therefore, a significant decrease in IgE antibody production may be ascribable to the decrease of IL-4 secretion in mice treated with HET. On the other hand, induction of interferon-α (IFN-α) in mice treated intraperitoneally with 250 mg/kg of HET was reported. IFN-α or interferon-γ blocked IL-4-induced IgE antibody production in a dose-dependent manner. Although the levels of IFN-α were not determined in this study, it is possible that the suppression of IgE antibody production was due to the elevation of the level of IFN-α.

In mice treated with OA plus HET, IgE and IgG1 antibody production on day 28 was suppressed compared to those with OA alone (Fig. 1), and especially the effect of HET was prominent on IgE. This phenomenon presumably depends on the different regulation of IgG1 and IgE antibody production by IL-4; i.e. IL-4 stimulated IgG1 secretion from resting mouse B in culture at lower concentrations than that required for IgE secretion, which reaches to a very high level. In mice treated with OA plus HET, IL-4 secrections from the splenic lymphocytes induced by stimulation with OA in vitro were approximately 60% of that of mice treated with OA alone (Fig. 2A). Therefore, the effect of HET on the level of IgG1 was not so conspicuous as compared to IgE.

In mice treated with OA plus HET, the levels of IL-4 gene expression in spleen or spleen cells were lower than those of the mice treated with OA alone (Fig. 6). These findings correspond to the significant suppression of IL-4 secretion from splenic lymphocytes by stimulation with OA in vitro (Fig. 2A).

In mice treated with OA plus HET, the proliferation of splenic lymphocytes induced by stimulation with OA or Con A, a T-cell mitogen, was significantly inhibited, but the stimulation with LPS, a B-cell mitogen, was barely affected (Fig. 4). Thus, HET may have an effect to suppress blastogenesis on T cells, but not on B cells. IL-4 promotes the proliferation of human T cells as a growth factor as well as IL-2. In mice treated with OA plus HET, the levels of IL-4 and IL-2 secretion from the splenic lymphocytes induced by stimulation with OA or Con A were lower than those of the mice treated with OA alone (Figs. 2 and 3). Therefore, HET was
assumed to inhibit cell proliferation induced by OA or Con A due to the decrease in IL-4 and IL-2 secretion from splenic T lymphocytes.

It is well known that chemical mediators, such as histamine etc., are released from basophils and mast cells by the binding of antigen, when the antibodies are located closely to each other on surface of those cells. Recently, Sihra et al. \(^{24}\) reported that high-affinity IgE receptor (FcεRI) expression, which is necessary for the induction of IgE-mediated allergic reaction, on the basophils of atopic patients and nonatopic control subjects, was significantly correlated with serum IgE concentrations. Lantz et al.\(^ {25}\) also reported that IgE concentration could be a major regulator of mouse basophil FcεRI expression in vivo. It has been reported that FcεRI expression on mast cells was also enhanced by an increase in IgE or IL-4.\(^ {26,27}\) It is possible that HET decreased FcεRI expression in response to the decreases of IgE and IL-4 levels, and in turn it decreased histamine release from basophils and mast cells.

In the present study, we have demonstrated the efficacy of HET in treating type I allergic diseases, such as asthma, since oral administration of HET suppressed IgE antibody production, IL-4 secretion, IL-4 gene expression, cell proliferation and histamine release closely related to type I allergy.

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