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Effect of orally administered Hochu-ekki-to, a Japanese herbal
medicine, on contact hypersensitivity caused by repeated
application of antigen

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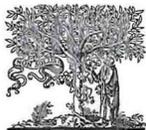
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Effect of orally administered Hochu-ekki-to, a Japanese herbal medicine, on contact hypersensitivity caused by repeated application of antigen

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Abstract

The effects of oral administration of Hochu-ekki-to (HET; bu-zhong-yi-qi-tang in Chinese), a traditional Japanese and Chinese herbal medicine, on chronic contact hypersensitivity were investigated. HET suppressed ear swelling due to chronic contact hypersensitivity caused by repeated application of 2,4,6-trinitro-1-chlorobenzene (TNCB). HET significantly suppressed not only increases in hapten-specific immunoglobulin E (IgE) and IgG1 titer due to repeated application of TNCB, but also total IgE and IgG1 concentration in the serum. Interleukin 4 (IL-4) level in inflamed ear tissue was significantly increased by repeated application of TNCB, and this increase in IL-4 level in the ear was significantly suppressed by oral administration of HET. Interferon γ (IFN- γ), IL-2, IL-5, IL-10 and IL-12 levels are not changed as much as IL-4 by TNCB and HET did not alter these cytokines as much as IL-4. These results suggest that oral administration of HET suppresses chronic contact hypersensitivity, and it can be assumed that the suppression of serum Ig E and Ig G1 and IL-4 in inflamed ear. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Hochu-ekki-to; Contact hypersensitivity; IL-4; IgE

1. Introduction

In allergic skin diseases, most inflammation is caused by chronic introduction of antigens into the skin. Atopic dermatitis is a common and distinctive disease associated with increased IgE production and positive immediate hypersensitivity. However, in patients with atopic dermatitis, repeated exposure to antigens through the skin is thought to contribute to the development of eczematous skin lesions, histo-

Abbreviations: HET, Hochu-ekki-to; TNCB, 2,4,6-trinitro-1-chlorobenzene; DTH, delayed-type hypersensitivity; TNP, 2,4,6-trinitrophenyl; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay.

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logically indistinguishable from those in allergic contact hypersensitivity, rather than cause the expected IgE-mediated urticarial reaction. Contact hypersensitivity is an acute inflammatory reaction elicited by epicutaneous applications of a sensitizing agent in animals previously sensitized with the same hapten. It is a T cell-mediated inflammatory response. Contact hypersensitivity is known as skin-specific delayed-type hypersensitivity (DTH) reaction associated with the infiltration of Th1 cells [1,2]. However, repeated elicitation of contact-sensitizing agents resulted in a shift in the time course of antigen-specific hypersensitivity responses from a typical delayed-type to an immediate-type response followed by a late reaction, elevated serum levels of antigen specific IgE [3]. Repeated exposure to antigens alters the balance of cytokine released locally, with a shift toward Th2-dominated responses [3,4]. These findings observed in chronic contact hypersensitivity in mice were accorded with those of patients with atopic dermatitis.

Traditional Japanese and Chinese herbal medicines (Japanese name; Kampo) are widely used for treating many kinds of acute and chronic diseases. Hochu-ekki-to (HET; bu-zhong-yi-qi-tang in Chinese) is a traditional herbal medicine which is a hot water extract of 10 different kinds of herbs. Clinically, HET has been widely used and covered by the governmental insurance to treat patients with reduced physical strength, cold constitution, anemia and anorexia. Recently, it has also been reported that HET improves the symptoms of atopic dermatitis [5].

Regarding to the effect of HET on allergic inflammation, it has been reported that HET suppressed the IgE mediated biphasic cutaneous reaction to 2,4-dinitrofluorobenzene (DNFB) [6], IgE production [7], histamine release from basophils [8] and eosinophils infiltration [9]. However, because most previous studies of HET on allergic response have been performed in animals after a few encounters with antigen, there were indistinctness as regards that effects of HET on chronic inflammation caused by a repeated exposure to antigen such as atopic dermatitis. Therefore, we studied the effect of HET on contact hypersensitivity induced by repeated application of 2,4,6-trinitro-1-chlorobenzene (TNCB).

The results in this study show that HET suppresses mainly chronic stages of contact hypersensi-

tivity. This suppression might be related to the immunological modification by HET, i.e. reduced serum IgE, IgG1 level and IL-4 production in the inflamed region.

2. Materials and methods

2.1. Drugs

Medicinal plants (crude drugs) used for the preparation of Hochu-ekki-to (HET) were the following. *Ginseng radix*, *Bupleuri radix*, *Glycyrrhizae radix*, *Zizyphi fructus* and *Astragali radix* were purchased from Uchida Wakan-Yaku (Tokyo, Japan). *Angelicae radix* and *Cimicifugae rhizoma* were purchased from Tochimoto Tenkaido (Osaka, Japan). *Atractylodes rhizoma*, *Zingiberis rhizoma* and *Aurantii nobilis pericarpium* were purchased from Tsumura and Co. (Tokyo, Japan). The *A. radix* used is classified into extra high-grade on the Japanese market, and the qualities of the other crude drugs are controlled by the Japanese Pharmacopoeia Thirteenth Edition. The HET used in this study was prepared according to the prescription book of Oriental Medicine Research Center of the Kitasato Institute. The components of the formulation of HET are listed in Table 1. The HET extract was prepared as follows. The mixture of the above mentioned crude drugs was decocted with 600 ml of boiling water for 40 min to half volume. The extracted solution was centrifuged at 6000 rpm for 20 min, and then the supernatant was filtered and frozen for storage at -20°C .

Table 1
Composition of Hochu-ekki-to

Medicinal plant	Amount (g)
<i>Astragali radix</i>	4.0
<i>Ginseng radix</i>	4.0
<i>Atractylodes rhizoma</i>	4.0
<i>Angelicae radix</i>	3.0
<i>Aurantii nobilis pericarpium</i>	2.0
<i>Bupleuri radix</i>	2.0
<i>Zizyphi fructus</i>	2.0
<i>Glycyrrhizae radix</i>	1.5
<i>Cimicifugae rhizoma</i>	1.0
<i>Zingiberis rhizoma</i>	0.5

2.2. Reagent and chemicals

2,4,6-Trinitro-1-chlorobenzene (TNCB) was purchased from Tokyo Kasei (Tokyo, Japan). 4-Methylumbelliferyl- β -D-galactoside and bovine serum albumin (BSA) was obtained from Sigma (St. Louis, MO). 2,4,6-Trinitrobenzene-sulfonic acid and *p*-nitrophenyl phosphate was obtained from Wako (Osaka, Japan).

Biotinylation of 2,4,6-trinitrophenyl (TNP)-bovine serum albumin was performed as follows. 2,4,6-Trinitrobenzene-sulfonic acid (40 mg) was added to 10 ml of 0.2 M borate buffer (pH 9.0) containing 2% BSA, and stirred for 18 h at room temperature. Following dialysis for 3 days against phosphate buffered saline (PBS), resulting TNP-BSA (6.5 mg/ml) was biotinylated with biotin (long arm)-NHS-water soluble® (Vector Laboratories, Burlingame, CA) under the condition of the supplier's instruction manuals.

2.3. Animals

Six to seven weeks old SPF male BALB/c (H-2^d) mice were purchased from Japan SLC (Shizuoka, Japan). The animals were maintained in a 24-h light and dark cycle (12 h of light, 12 h of darkness) and controlled temperature (22±1 °C). They had free access to standard laboratory chow (CE-2, from CLEA Japan) and water. There were three groups of mice, one is treated with acetone only (vehicle alone), one is treated with TNCB and one is treated with TNCB and HET. HET was administered orally by free access to drinking water everyday from the day of sensitization (day -7). The control mice were provided with tap water alone as a vehicle. The average dose of HET was estimated to be 1000 mg/kg/day which is 20-fold of the clinical dose used for humans. During the experiments, there was no difference in body weight, behavior or motor deficit between the groups.

2.4. Procedure for contact hypersensitivity by repeated application of TNCB

The sensitization and repeated elicitation was performed according to the procedure of Kitagaki et al. [3,4]. Briefly, BALB/c mice were sensitized by single epicutaneous application with 20 μ l of 1% TNCB

solution to the right ear 7 days before the first elicitation. Then, 20 μ l of 1% TNCB solution was repeatedly applied to the sensitized right ear from day 0 to day 18 at 2-day intervals. Ear thickness was measured by a dial thickness gauge (Ozaki Seisakusho, Tokyo, Japan) and expressed as the increase in thickness from before sensitization.

2.5. Antibodies and standards for enzyme-linked immunosorbent assay

The antibodies used for enzyme-linked immunosorbent assay (ELISA) were anti-IL-2 (clone ES6-1A12), biotin-labeled anti-IL-2 (JES6-5H4), anti-IL-4 (11B11), biotin-labeled anti-IL-4 (BVD6-24G2), anti-IL-5 (TRFK5), biotin-labeled anti-IL-5 (TRFK4), anti-IL-10 (JES5-2A5), biotin-labeled anti-IL-10 (SXC-1), anti-IL-12 (C15.6), biotin-labeled anti-IL-12 (C17.8), anti-IFN- γ (R4-6A2), biotin-labeled anti-IFN- γ (XMG1.2), anti-IgE (R35-72), biotin-labeled anti-IgE (R35-92), anti-IgG1 (A85-3), biotin-labeled anti-IgG1 (A85-1), anti-IgG2a (R11-89), biotin-labeled anti-IgG2a (R19-15) and anti-IgG2b (R9-91), biotin-labeled anti-IgG2b (R12-3). All used antibodies were purchased from Pharmingen (San Diego, CA). For standard curve, recombinant mouse IL-2, IL-4, IL-5, IL-10, IL-12, IFN- γ and purified anti-trinitrophenyl IgE were also purchased from Pharmingen, and purified mouse IgG1, IgG2a and IgG2b were obtained from Zymed Laboratories (San Francisco, CA).

2.6. Analysis of cytokine production in inflammatory regions

Cytokine production in the inflamed region was determined by ELISA [10]. Forty-eight hours after application of TNCB, mice were anesthetized with ether and sacrificed by the cervical dislocation, then the ear was excised and homogenized with 30-fold volume of PBS containing 0.1% Tween-20. All the procedures were performed according to the guideline for the care and use of laboratory animals of the institute. The homogenates were frozen in liquid nitrogen, thawed in a 37 °C water bath, sonicated for 10 s, and centrifuged for 10 min at 12,000 \times g. Supernatants were subjected to ELISA for cytokines. A solution (2 μ g/well) of captured antibody in 0.1 M

bicarbonate buffer (pH 8.6) was added to microtiter plates for ELISA (ICN Biomedicals, Aurora, OH) for overnight incubation at 4 °C. Unbound antibody was removed by washing with PBS containing 0.05% Tween 20 (PBS-Tween) four times. The plate was further incubated with 1% skim milk in PBS at room temperature for 2 h (blocking). Samples were added to the first antibody-coated well at a volume of 50 µl/well, and the plates were incubated overnight at 4 °C. For the standard curve, titrated recombinant cytokine was used. After the plate was washed four times with PBS-Tween, 100 µl of the biotin-labeled anti-cytokine-detecting antibody (2 µg/ml) was added to the wells, and the plate was incubated for 1 h at room temperature. Then the plate was washed six times with PBS-Tween. Streptavidin-β-galactosidase (Life Technologies, Gaithersburg, MD) diluted with 1% skim milk (1:3000) was added to each well and the plates were then incubated at room temperature for 30 min. After washing the wells with PBS-Tween for eight times, each well was incubated with 200 µl of substrate solution (containing 2% 4-methyl-umbelliferyl-β-D-galactoside, 0.01% BSA and 2 mM MgCl₂). The fluorescence intensity was measured by Fluoroskan II (Labsystems Oy, Helsinki, Finland) at an excitation wavelength of 544 nm and an emission wavelength of 590 nm. The delta soft II program for Macintosh (ver 4.13 FL, BioMetallics, Princeton, NJ) was used for data evaluation. The amount of cytokine in each sample was then calculated using the standard curve on each plate, respectively.

2.7. Determination of hapten-specific IgE titer and total IgE concentration in serum

Mice were bled from retro orbital plexus using capillary glass micropipette (Drummond Scientific, Broomall, PA) under ether anesthesia 48 h after application of TNCB and sera were obtained. The sera were stored at -20 °C until analysis. Hapten-specific IgE titer in mouse serum was measured by captured ELISA. Briefly, EIA plate was coated with anti-IgE antibody (clone 35-72) (2 µg/ml) and blocked with 1% skim milk in PBS. One hundred microliter of serum sample diluted with 1% skim milk was added to each well and plates were incubated overnight at 4 °C. After washing, 100 µl of diluted biotinylated TNP-BSA was added to each well and incubated for

1 h at room temperature. After extensive washing with 1% skim milk, 100 µl of diluted streptavidin-β-galactosidase were added. Following final incubation for 30 min, the plate was washed and the substrate solution, containing 4-methyl-umbelliferyl-β-D-galactoside, was added. The fluorescence intensity was measured as described above.

Total serum IgE (tIgE) was measured using the same ELISA method for cytokines as described above. Anti-mouse IgE (R35-72) and biotin-labeled anti-mouse IgE (R35-92) were used capture and detection antibody, respectively.

2.8. Determination of hapten-specific and total IgG subclass concentration in serum

Hapten-specific IgG subclass (IgG1, IgG2a and IgG2b) titer in mouse serum was measured by ordinary EIA methods. Briefly, the EIA plate was coated with TNP-BSA (0.16 µg/well) in 0.1 M bicarbonate buffer (pH 8.6) overnight at 4 °C, and then blocked with 1% skim milk in PBS. The diluted samples were added (50 µl) to each well, and incubated overnight at 4 °C. After washing, 100 µl of alkaline phosphatase labeled with murine IgG subclass specific antibody (2 µg/ml) were added, and incubated for 1 h at room temperature. The plate was washed, then the substrate solution (1 mg of *p*-nitrophenylphosphate disodium salt in 1 ml of diethanolamine buffer, pH 9.8) was added. Subsequently the absorbance at 405 nm was measured using a microplate reader (Bio-Rad, Model 250). Because of suitable standards were not available, data were compared to the value of the absorbance obtained at 405 nm.

Total IgG subclasses in serum were measured by ELISA. Briefly, the EIA plate was coated with a solution (2 µg/well) of murine IgG subclass specific antibody (capture antibody) incubated overnight at 4 °C, and then blocked with 1% skim milk in PBS. The diluted sample was added to the first antibody-coated well at a 50 µl/well, and the plates were again incubated overnight at 4 °C. For the standard curve, titrated standard protein was used. After washing, 100 µl of the alkaline phosphatase-labeled anti-murine IgG subclass antibody (detection antibody, 2 µg/ml) was added to the well, and the plate was incubated for 1 h at room temperature. The plate was washed, substrate solution (1 mg of *p*-nitrophenylphosphate disodium

salt in 1 ml of diethanolamine buffer, pH 9.8) was added, and subsequently the absorbance spectrum was measured as described above.

2.9. Statistical analysis

To define statistical significant difference, data were subjected to repeated measure analysis of variance (repeated measure ANOVA) and Welch's test. *p* Values less than 0.05 were considered to be statistically significant. The analysis of repeated measure ANOVA was performed using a personal computer with the StatView-J program for Macintosh (ver 5.0, SAS Institute, Cary, NC), and Welch's test was performed with DA Stats (ver 1.0, freeware soft, copyright® 1993 by Dr. O. Nagata) after the variances of data were examined using the *F*-test.

3. Results

3.1. Effect of HET on ear swelling responses

Repeated application of TNCB induced a dramatically increase in the total ear thickness, and this increment reached a plateau at day 12 (Fig. 1), whereas repeated treatment with acetone alone as a vehicle had no detectable changes. By oral administration of HET, the increase in ear thickness was significantly suppressed after day 12 (Fig. 1). Comparison of TNCB-treated with and without HET groups for the time period from day 0 to day 18 showed a difference with statistical significance ($P=0.024$, repeated measure ANOVA). These results suggest that HET suppressed mainly chronic stages of contact hypersensitivity. On day 18, the ear swelling response appeared within 30 min after elicitation of TNCB, reached a peak at 3–6 h and then decreased gradually to basal line within 24 h (Fig. 2). The time course of ear swelling response showed that oral administration of HET suppressed the ear swelling significantly at all time points measured mainly due to the baseline difference (Fig. 2).

3.2. Effect of HET on hapten specific IgE titer and IgE concentration in serum

As IgE is thought to be important in the pathogenesis of contact hypersensitivity reaction, we next

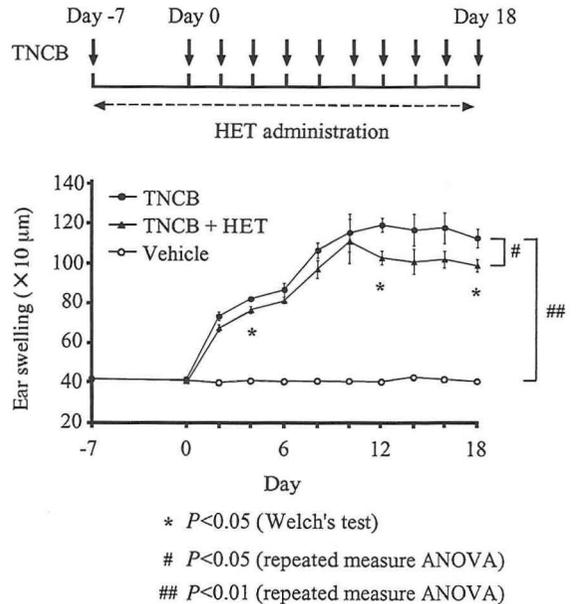


Fig. 1. Effect of HET on ear swelling induced by repeated elicitation of TNCB. Mice were sensitized on the right ear by epicutaneous application of 20 μl of 1% TNCB solution 7 days before first elicitation, and then the mice were repeatedly treated with TNCB from day 0 at 2 days interval. The ear thickness was measured 48 h after elicitation (just before each elicitation). Repeated treatment with vehicle (acetone) serves as control (Vehicle; open circle). HET was administered orally everyday with drinking water from the day of sensitization (day -7) to day 18. Data were represented as mean of ear thickness ($\times 10 \mu\text{m}$) \pm S.E. ($n=5$). Asterisk shows the significant difference between the ear thickness of the TNCB-treated mice with (TNCB + HET; closed triangle) and without HET (TNCB; closed circle) on a single day by Welch's test ($P < 0.05$). Sum of the thickness from day 0 to day 18 was also analyzed by repeated measure ANOVA. Statistical significance was observed between the ear thickness of the TNCB-treated mice with (TNCB + HET; closed triangle) and without HET (TNCB; closed circle) or vehicle only (Vehicle) group, # $P < 0.05$, ## $P < 0.01$.

examined the effect of HET on hapten specific IgE (sIgE) titer and total IgE (tIgE) concentration in the serum. The sIgE and tIgE were measured by ELISA at day 0, day 6 and day 18, respectively. The slightly increased levels of tIgE and sIgE by sensitization of TNCB were observed at day 0, and this increment of tIgE and sIgE levels were enhanced by repeated application of TNCB in chronic stages (at day 18) (Fig. 3). On day 0 and day 6, no measurable changes in tIgE and sIgE levels in serum were observed between TNCB-treated with HET and without HET

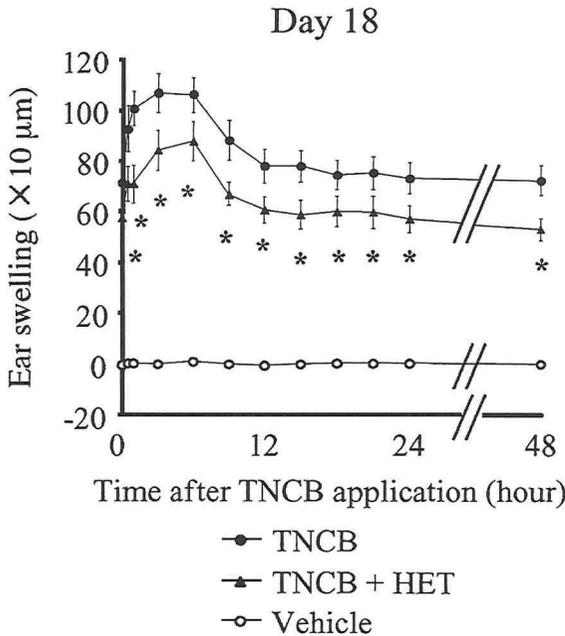


Fig. 2. Effects of HET on ear swelling response. Mice were sensitized on the right ear by epicutaneous application of 20 μl of 1% TNCB solution 7 days before first elicitation, and then the mice were repeatedly treated with TNCB from day 0 at 2 days interval. Repeated treatment with acetone serves as vehicle control. HET was administered orally everyday with drinking water from the day of sensitization (day -7). Mice were administrated HET orally with drinking water everyday from the day of sensitization (day -7). The time course of ear swelling response was measured at several time points as indicated in figure after elicitation on day 18. The ear swelling were expressed as the increase in thickness from the ear thickness at day 0. Data were represented as mean of ear swelling (×10 μm)±S.E. (n=5). Statistical analysis between the ear thickness of the TNCB-treated mice with (TNCB+HET; closed triangle) and without HET (TNCB; closed circle) on a single day was performed by Welch's test (*P<0.05). Repeated treatment with vehicle (acetone) serves as control (Vehicle; open circle).

groups. However, notable changes in tIgE and sIgE levels by oral administration of HET were observed in chronic stage. Oral administration of HET remarkably suppressed both tIgE and sIgE level on day 18 (Fig. 3).

3.3. Effect of HET on IgG subclass

It has been reported that not only IgE but also IgG1 contributes to allergic reactions [11]. Therefore, we examined the effect of oral administration of HET on

hapten-specific and total IgG1 concentration in serum. A slightly increased level of hapten specific IgG1 (sIgG1) titer by sensitization of TNCB was seen in the serum on day 0, and remarkable increase in both sIgG1 and tIgG1 was observed by after repeated application of TNCB (Fig. 4). No measurable differences in tIgG1 and sIgG1 levels in the serum were observed between TNCB-treated with HET and with-

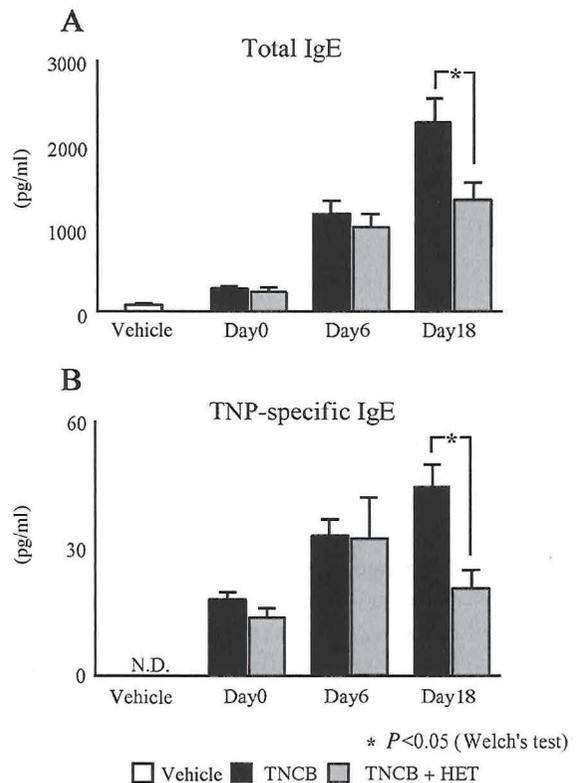


Fig. 3. Effect of HET on total IgE (A) and TNP specific IgE (B) concentration in serum. Mice were sensitized on the right ear by epicutaneous application of 20 μl of 1% TNCB solution 7 days before first elicitation, and then the mice were repeatedly treated with TNCB from day 0 at 2 days interval. HET was administered orally everyday with drinking water from the day of sensitization (day -7). Mice were administrated HET orally with drinking water everyday from the day of sensitization (day -7). Mice were bled 48 h after elicitation of TNCB on day 0, day 6 and day 18, respectively, and resulting sera were subjected to ELISA for total IgE and for TNP-specific IgE as described in Materials and Methods. Each value represented as mean ± S.E. (n=5). Statistical analysis between the TNCB-treated mice with (hatched bar) and without HET (filled bar) by Welch's test (*P<0.05). Open bars; vehicle only group.

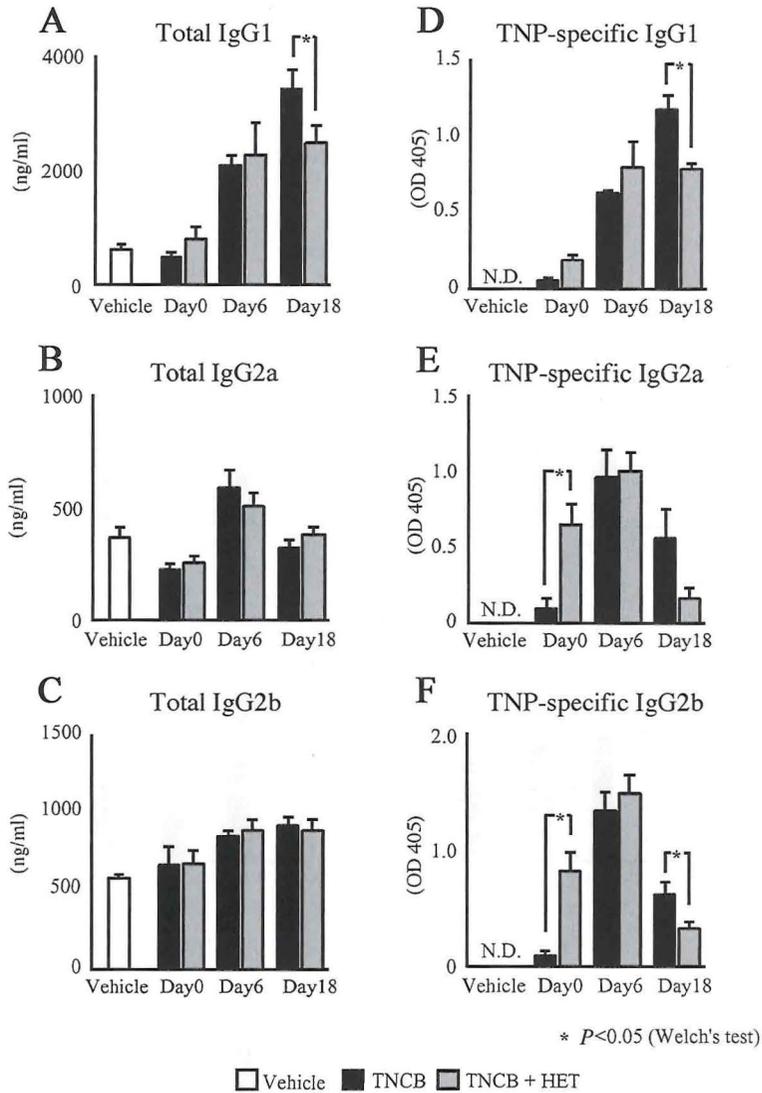


Fig. 4. Effect of HET on total-IgG1 (A), -IgG2a (B), -IgG2b (C) and TNP-specific-IgG1 (D), -IgG2a (E), -IgG2b (F) subclasses in serum. Mice were sensitized on the right ear by epicutaneous application of 20 μ l of 1% TNCB solution 7 days before first elicitation, and then the mice were repeatedly treated with TNCB from day 0 at 2 days interval. HET was administered orally everyday with drinking water from the day of sensitization (day -7). Mice were bled 48 h after elicitation of TNCB on day 0, day 6 and day 18, respectively, and sera were subjected to ELISA as described in Materials and Methods. Each value represented as mean \pm S.E. ($n = 5$). Open bar; vehicle only group, filled bar; TNCB-treated group without HET, hatched bar; TNCB-treated group with HET. Statistical analysis between the TNCB-treated mice with (hatched bar) and without HET (filled bar) was performed by Welch's test ($*P < 0.05$).

out HET groups on day 0 and day 6. However, definite changes in tIgG1 and sIgG1 level by oral administration of HET were observed in the chronic stage. HET significantly suppressed increases of both tIgG1 and sIgG1 levels on day 18 (Fig. 4).

In addition to IgG1, IgG2a and IgG2b levels in the serum were also measured. sIgG2a and sIgG2b were detected 1 week after sensitization (day 0), and were increased by repeated application of TNCB (Fig. 4). Oral administration of HET significantly

increased sIgG2a and sIgG2b concentrations in the serum on day 0 ($P < 0.001$). However, when compared to the TNCB-treated without HET group in chronic stage on day 18, sIgG2b were decreased ($P = 0.049$) (Fig. 4).

3.4. Effect of HET on cytokine productions in inflammatory regions

The cytokine secretion is the focus of much immunological research at present, with the aim to identify

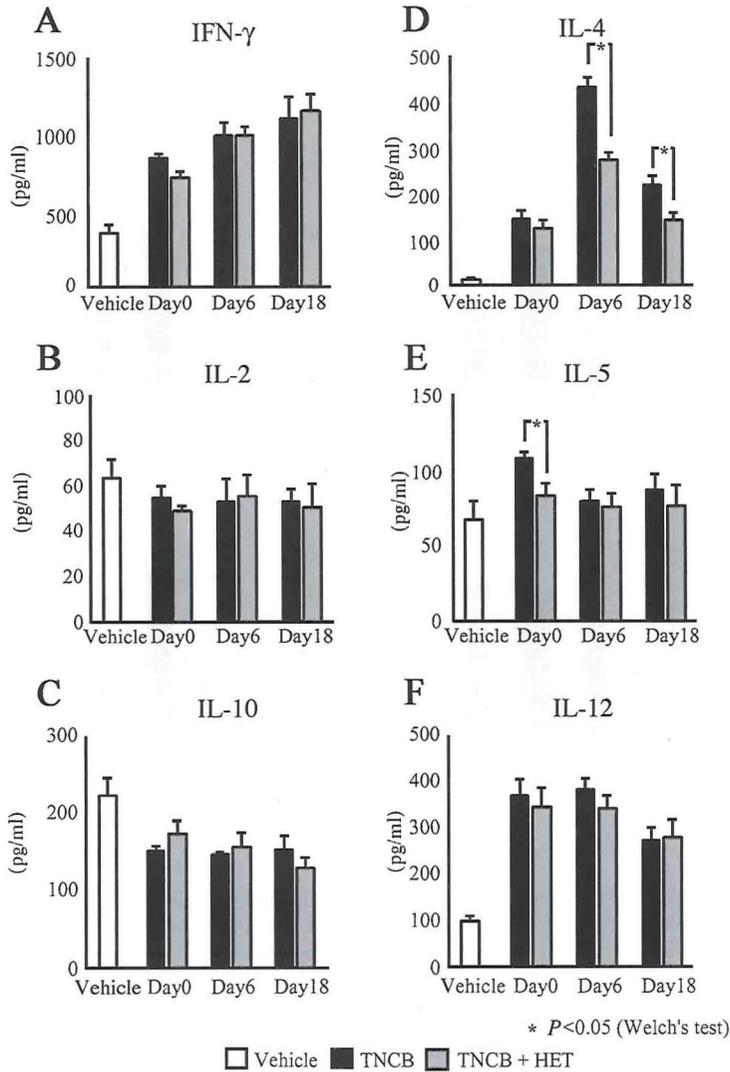


Fig. 5. Effect of HET on cytokine concentrations in inflammatory regions. Mice were sensitized on the right ear by epicutaneous application of 20 μ l of 1% TNCB solution 7 days before first elicitation, and then the mice were repeatedly treated with TNCB from day 0 at 2 days interval. HET was administered orally everyday with drinking water from the day of sensitization (day -7). Forty-eight hours after application of TNCB on day 0, day 6 and day 18, the inflamed ear was excised, homogenized and centrifuged, and then supernatants were subjected to ELISA for cytokines as described in Materials and Methods. Each value represented as mean \pm S.E. ($n = 5$). Open bar; vehicle only group, filled bar; TNCB-treated group without HET, hatched bar; TNCB-treated group with HET. Statistical analysis between the TNCB-treated mice with (hatched bar) and without HET (filled bar) was performed by Welch's test (* $P < 0.05$).

and analyze cellular and molecular parameters that lead to understand inflammatory immune response. The effects of orally administered HET on cytokine production in inflamed regions were investigated. The cytokines tested were IFN- γ , IL-2, IL-4, IL-5, IL-10 and IL-12. By application of TNCB, notable increase in IL-4 level in the inflamed ear was observed (Fig. 5). Administration of HET significantly reduced IL-4 level on day 6 and day 18 (Fig. 5D). No measurable effects by administration of HET on the amount of the other cytokines tested such as IFN- γ , IL-2, IL-5, IL-10 and IL-12 were observed in chronic stage (Fig. 5).

4. Discussion

We have shown that orally administered HET suppresses the ear swelling response in chronic contact hypersensitivity caused by repeated application of TNCB. In this study, HET was administered to mice at a dose of 1000 mg/kg/day, which is equal to 20-fold for the clinical dose applied in human. It is widely known that human have a low metabolic ability on drugs [12]. Inversely, drugs are metabolized in mice more quickly than that of human. Indeed, it was reported that the hepatic clearance of drugs in humans was approximately one-seventh of that of other mammals including mice [13]. Furthermore, it was reported that when compared renal clearance rate between human and mice, clearance of mice is about 10-fold greater than that of human [14,15]. Therefore, we used relatively high dosage in this study.

In our data, increase of antigen-specific IgE levels by repeated application of TNCB was observed, and this result was well in accordance with previous report [3]. In addition to antigen-specific IgE, remarkable increases in antigen-specific IgG1, total-IgE and -IgG1 in serum were observed by repeated application of TNCB. IgE and IgG1 are the only immunoglobulin isotypes that can elicit active- and passive-anaphylactic reaction through binding to Fc ϵ RI and Fc γ RIII, respectively, on mast cells [11,16]. Therefore not only antigen specific-IgE but also antigen specific-IgG1 might contribute to TNCB induced chronic contact hypersensitivity.

Repeated application of TNCB induced increased IL-4 in inflamed tissue. IL-4 is one of Th2 cytokines which promotes immunoglobulin class switching to

IgE and IgG [17,18], and stimulates immunoglobulin secretion of B cells [19]. The increase in IL-4 levels by repeated application of TNCB was significantly suppressed by oral administration of HET. Therefore, it is presumed that the reduction of both, antigen specific-IgE and -IgG1, might be partly explained by the reduction of IL-4 by HET as a possible mode of action.

Evidence exists that the Th1 and Th2 types of immune response are reciprocally regulated in vivo [20–22]. Especially, IFN- γ , which is mainly produced by Th1 cells, inhibits the proliferation of Th2 cells and their IL-4 production [23,24]. It is noteworthy that the IL-4 concentration in inflamed tissue was significantly reduced by oral administration of HET, whereas no significant changes of IFN- γ concentration were observed. These results suggest that the inhibitory effect of HET on IL-4 production was not due to Th1 activation, but it is possible that oral administration of HET might directly inhibit specifically the IL-4 production, because other Th2 cytokines like IL-5 or IL-10 were not affected by HET administration. It is important to ask which population of cells modulated the function by HET. Thus, in order to clarify the mode of action of HET, it is important to determine the source of cytokines. It has been reported that mast cells and small mononuclear cells were observed as IL-4 positive cells in the ear after repeated application of TNCB [4]. IL-4 is produced by various cells such as T cells, NKT cells, mast cells and basophiles [22,25–27]. Recently, B cells were classified into Be1 and Be2 subsets according to their cytokine secretion pattern similarly to Th1 and Th2 [28]. Be2 has been shown to secrete significant amount of IL-4. In order to provide direct proof for cytokine producing cells, in situ hybridization for cytokine mRNA and/or multi-stained immunohistochemical study are needed to be done. The mechanism of HET on inhibition of increase in IL-4 after antigen exposure is not known at present. Various mechanisms are proposed for the modulation of immune responses observed after the oral administration of HET. In order to clarify the mode of action, fractionation and characterization of the active substances in HET are also now underway, and details of active substances as well as mode of actions will be described in future.

Increased level of hapten specific-IgG2a and -IgG2b were observed by oral administration on day 0. The

mechanisms of this increase of antigen specific-IgG2a and -IgG2b level are also not known at present time, however it has been reported that Ginseng extract enhances IgG2a, and reduces IgG1 production in a rat model of chronic *Pseudomonas aeruginosa* pneumonia [29]. As *G. radix* is one of the main components, in HET, it might be due to effect of Ginseng. As mentioned above, HET suppresses the ear swelling response in chronic stage, it is presumed that this increase in hapten specific-IgG2a and -IgG2b on day 0 might not contribute to the suppression of swelling response by HET.

Atopic dermatitis is a chronic and relapsing inflammatory skin disease associated with increased IgE production leading to the idea that the immediate-type hypersensitivity is involved. Repeated exposure to antigen is thought to contribute to the development of inflammatory skin lesions [30,31]. It has been reported that the chronic contact hypersensitivity in the mice used in this study closely resembles to atopic dermatitis in humans [3,4]. Clinically, HET has been used to treat atopic dermatitis, and beneficial effect was reported [5]. The suppression of the hypersensitivity reaction together with reduction of IgG1, IgE and IL-4 production may explain a part of the clinical observation and our findings suggest that the further application of HET for the treatment of allergic disease caused by repeated exposure to antigen such as atopic dermatitis in human beings.

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References

- [1] Grewe M, Walther S, Gyufko K, Czech W, Schopf E, et al. Analysis of the cytokine pattern expressed in situ in inhalant allergen patch test reactions of atopic dermatitis patients. *J Invest Dermatol* 1995;105:407–10.
- [2] Hamid Q, Boguniewicz M, Leung DY. Differential in situ cytokine gene expression in acute versus chronic atopic dermatitis. *J Clin Invest* 1994;94:870–6.
- [3] Kitagaki H, Fujisawa S, Watanabe K, Hayakawa K, Shiohara T. Immediate-type hypersensitivity response followed by a late reaction is induced by repeated epicutaneous application of contact sensitizing agents in mice. *J Invest Dermatol* 1995;105:749–55.
- [4] Kitagaki H, Ono N, Hayakawa K, Kitazawa T, Shiohara T. Repeated elicitation of contact hypersensitivity induces a shift in cutaneous cytokine milieu from a T helper cell type 1 to a type 2 profile. *J Immunol* 1997;159:2484–91.
- [5] Kobayashi H, Mizuno N, Ishii M, Takahashi K, Yamamoto I. Clinical effects of Hochu-ekki-to in patients with atopic dermatitis (III). Influence of the administration on serum cytokines levels. *J Tradit Med* 1998;15:310–1.
- [6] Tahara E, Satoh T, Watanabe C, Shimada Y, Terasawa K, et al. Effect of Kampo medicines on IgE-mediated biphasic cutaneous reaction in mice. *J Tradit Med* 1998;15:100–8.
- [7] Kaneko M, Kishihara K, Kawakita T, Nakamura T, Takimoto H, et al. Suppression of IgE production in mice treated with a traditional Chinese medicine, bu-zhong-yi-qi-tang (Japanese name: hochu-ekki-to). *Immunopharmacology* 1997;36:79–85.
- [8] Suzuki T, Takano I, Nagai F, Fujitani T, Ushiyama K, et al. Suppressive effects of Hochu-ekki-to, a traditional Chinese medicine, on IgE production and histamine release in mice immunized with ovalbumin. *Biol Pharm Bull* 1999;22:1180–4.
- [9] Kaneko M, Kawakita T, Nomoto K. Inhibition of eosinophil infiltration into the mouse peritoneal cavity by a traditional Chinese medicine. Bu-zhong-yi-qi-tang (Japanese name: Hochu-ekki-to). *Immunopharmacol Immunotoxicol* 1999;21:125–40.
- [10] Ferguson TA, Dube P, Griffith TS. Regulation of contact hypersensitivity by interleukin 10. *J Exp Med* 1994;179:1597–604.
- [11] Miyajima I, Dombrowicz D, Martin TR, Ravetch JV, Kinet JP, et al. Systemic anaphylaxis in the mouse can be mediated largely through IgG1 and Fcγ(RIII): assessment of the cardiopulmonary changes, mast cell degranulation, and death associated with active or IgE- or IgG-dependent passive anaphylaxis. *J Clin Invest* 1997;99:915–25.
- [12] Brodie BB. Of mice, microsomes and man. *Pharmacologist* 1964;6:12–26.
- [13] Boxenbaum H. Interspecies variation in liver weight, hepatic blood flow, and antipyrine intrinsic clearance: extrapolation of data to benzodiazepines and phenytoin. *J Pharmacokinet Biopharm* 1980;2:165–76.
- [14] Adolph EF. B cell stimulatory factor-1/interleukin-4 mRNA is expressed by normal and transformed mast cells. Quantitative relations in the physiological constitutions of mammals. *Science* 1949;109:579–85.
- [15] Dedrick RL, Bischoff KB, Zaharko DS. Interspecies correlation of plasma concentration history of methotrexate (NSC-740). *Cancer Chemother Rep Part 1* 1970;54:95–101.
- [16] Becker EL. Nature and classification of immediate-type allergic reactions. *Adv Immunol* 1971;13:267–313.
- [17] Lebman DA, Coffman RL. Interleukin 4 causes isotype switching to IgE in T cell-stimulated clonal B cell cultures. *J Exp Med* 1988;168:853–62.
- [18] Kepron MR, Chen YW, Uhr JW, Vitetta ES. IL-4 induces the specific rearrangement of gamma 1 genes on the expressed and

- unexpressed chromosomes of lipopolysaccharide-activated normal murine B cells. *J Immunol* 1989;143:334–9.
- [19] Paul WE. Interleukin-4: a prototypic immunoregulatory lymphokine. *Blood* 1991;77:1859–70.
- [20] Heinzel FP, Sadick MD, Holaday BJ, Coffman RL, Locksley RM. Reciprocal expression of interferon γ or IL-4 during the resolution or progression of murine leishmaniasis: evidence for distinct helper T cell subsets. *J Exp Med* 1989;169:59–72.
- [21] Romagnani S. Human Th1 and Th2 subsets: doubt no more. *Immunol Today* 1991;12:256–7.
- [22] Mosmann TR, Coffman RL. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu Rev Immunol* 1987;7:145–73.
- [23] Gajewski TF, Schell SR, Nau G, Fitch FW. Regulation of T-cell activation: differences among T-cell subsets. *Immunol Rev* 1989;111:79–110.
- [24] Stout RD, Bottomly K. Antigen-specific activation of effector macrophages by IFN- γ producing (TH1) T cell clones. Failure of IL-4-producing (TH2) T cell clones to activate effector function in macrophages. *J Immunol* 1989;142:760–5.
- [25] Chen H, Paul WE. Cultured NK1.1+ CD4+ T cells produce large amounts of IL-4 and IFN- γ upon activation by anti-CD3 or CD1. *J Immunol* 1997;159:2240–9.
- [26] Brown MA, Pierce JH, Watson CJ, Falco J, Ihle JN, et al. B cell stimulatory factor-1/interleukin-4 mRNA is expressed by normal and transformed mast cells. *Cell* 1987;50:809–18.
- [27] Seder RA, Paul WE, Ben-Sasson SZ, LeGros GS, Kagey-Sobotka A, et al. Production of interleukin-4 and other cytokines following stimulation of mast cell lines and in vivo mast cells/basophils. *Int Arch Allergy Appl Immunol* 1991;94:137–40.
- [28] Harris DP, Haynes L, Sayles PC, Duso DK, Eaton SM, et al. Reciprocal regulation of polarized cytokine production by effector B and T cells. *Nat Immunol* 2001;1:475–82.
- [29] Song Z, Kharazmi A, Wu H, Faber V, Moser C, et al. Effects of ginseng treatment on neutrophil chemiluminescence and immunoglobulin G subclasses in a rat model of chronic *Pseudomonas aeruginosa* pneumonia. *Clin Diagn Lab Immunol* 1998;5:882–7.
- [30] Cooper KD. Atopic dermatitis: recent trends in pathogenesis and therapy. *J Invest Dermatol* 1994;102:128–37.
- [31] Ohmen JD, Hanifin JM, Nickoloff BJ, Rea TH, Wyzzykowski R, et al. Overexpression of IL-10 in atopic dermatitis. Contrasting cytokine patterns with delayed-type hypersensitivity reactions. *J Immunol* 1995;154:1956–63.

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