Immunopharmacological properties of Oren-gedoku-to (a Kampo medicine, Huang-Lian-Jie-Du-Tang) on contact hypersensitivity reaction in mice

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Abstract

We investigated the effects of Oren-gedoku-to (Huang-Lian-Jie-Du-Tang), a Kampo medicine, on DNFB-induced contact hypersensitivity (CHS) response in mice in order to further clarify the immunopharmacological properties of this formulation. 1) Administration of Oren-gedoku-to decreased the magnitude of ear swelling in the CHS response and shortened the affected period. The inhibitory effect on ear swelling was observed even when Oren-gedoku-to was given orally with different timing schedules. 2) The expressions of mRNAs for CD8, IFN-γ and TNF-α in the ear of Oren-gedoku-to-treated mice were markedly decreased 24 h after the challenge. 3) The number of skin-draining regional lymph node cells (LNCs), $CD4^+$ T cells and $CD8^+$ T cells was decreased without affecting the ratio of $CD8^+$/$CD4^+$ T cells. 4) Oren-gedoku-to resulted in a marked impairment of the hapten-specific development of LNCs. These results suggest that the suppressive effect of Oren-gedoku-to on ear swelling was partly caused by the suppression of lymphocyte proliferation.

Key words Oren-gedoku-to, CHS, DNFB.

Abbreviations ABS, absorbance; AD, atopic dermatitis; BrdU, 5-bromo-2'deoxyuridine; CHS response, contact hypersensitivity response; DNBS, 2,4-dinitrobenzenesulfonate; DNFB, 2,4-dinitrofluorobenzene; IFN-γ, Interferon gamma; IgE, immunoglobulin E; Ji-zuso-ippo (Zhi-Tou-Chuang-Yi-Fang), 治頭瘡一方; LC, Langerhans' cell; LN, lymph node; LNC, LN cell; LTB4, Leukotriene B4; mRNA, messenger ribonucleic acid; Oren-gedoku-to (Huang-Lian-Jie-Du-Tang), 黄連解毒湯; PC, picryl chloride; RT-PCR, reverse transcriptase-polymerase chain reaction; Sho-saiko-to (Xiao-Chai-Hu-Tang), 小柴胡湯; Sho-sei-ryu-to (Xiao-Qing-Long-Tang), 小青竜湯; Th, helper T; TNF-α, tumor necrosis factor-alpha; Tokaku-joki-to (Tao-He-Cheng-Qi-Tang), 桃核承気湯.

Introduction

A recent increase in the incidence of chronic allergic diseases including atopic dermatitis (AD) has been reported.[1,2] More than 80% of the patients with AD showed elevated levels of serum IgE,[3] but a close relationship between allergic responses and the pathogenesis of the skin lesions of AD is not yet clear. The infiltration of activated T cells into skin lesions and the dynamic production of T cell-derived cytokines have been shown in AD patients.[4-6] In addition to the marked production of T helper (Th) 2 cytokines in AD patients,[4] chronic skin lesions and late-phase allergic responses are characterized by Th1 cytokines.[5,6] Therefore, it should be important for therapeutic treatment of AD to control the responses characterized by a Th1 cytokine profile such as the contact hypersensitivity (CHS) response, as well as a Th2 cytokine profile such as IgE production.

The mainstay of therapy for AD remains a topical application of steroids. However, various treatment modalities including Kampo medicines have been used to

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improve AD effectively. In clinical settings, Kampo medicines have been widely used for allergic and inflammatory diseases. The mechanisms of their action have been investigated,[7-12] but their details are still largely unknown. We previously reported that some Kampo formulations and synthetic anti-allergic agents inhibited the IgE-mediated triphasic cutaneous reaction of immediate phase response (IPR), late phase response (LPR) and very late phase response (vLPR), peaking at 1 h, 24 h and 8 days after the challenge, respectively.[13-15] The inhibitory effects of the Kampo formulations on the triphasic cutaneous reaction were divided into several groups according to the efficacies for IPR/LPR/vLPR.[14,15] For instance, the group consisting of formulations such as Tokaku-joki-to, Ji-zuso-ippo, Sho-sei-ryu-to and Sho-saiko-to significantly inhibited IPR, LPR and vLPR (*i.e.* +/+/+ groups that showed inhibitory effects against the triphasic response), similar to the effect of prednisolone as a positive control.[14] Since a platelet activating factor (PAF) receptor antagonist (Y-24180) and a leukotriene B4 (LTB4) receptor antagonist (ONO-4057) were effective at inhibiting both LPR and vLPR,[15] Kampo formulations in the +/+/+ and/or -/+/+ groups are expected to show such anti-PAF and LTB4 activities.[15]

Oren-gedoku-to is one of the most popular Kampo formulations against atopic dermatitis, and is administered to patients with eczema or pruritus due to relative Yang-excess of the middle or exterior of the body, according to the Kampo diagnosis system.[16] On the other hand, oral administration of Oren-gedoku-to inhibited the picryl chloride (PC)-elicited CHS response characterized by a Th1 cytokine.[12] In the present study, we investigated the immunopharmacological effects of Oren-gedoku-to on murine CHS response in detail.

Materials and Methods

Mice: Female BALB/c mice were purchased from Japan SLC Inc (Hamamatsu, Japan) and maintained in the Laboratory for Animal Experiments, Institute of Natural Medicine, Toyama Medical and Pharmaceutical University. All mice were used at 7 to 10 weeks of age. This study was conducted in accordance with the standards outlined in the Guidelines for the Care and Use of Laboratory Animals of Toyama Medical and Pharmaceutical University.

Table I Crude drug composition of Oren-gedoku-to

Crude drug (Japanese name)	Composition (g)	Botanical origin
Scutellariae Radix (Ogon)	3.0	*Scutellaria baicalensis* GEORGI
Coptidis Rhizoma (Oren)	2.0	*Corptis japonica* MAKINO
Gardeniae Fructus (Sanshishi)	2.0	*Gardenia jasminoides* ELLIS
Phellodendri Cortex (Obaku)	1.5	*Phellodendron amurense* RUPRECHT

Oren-gedoku-to: Oren-gedoku-to (Huang-Lian-Jie-Du-Tang; lot no. 2000015010, Tsumura Co., LTD., Tokyo, Japan) is composed of four crude drugs (Table I), the quality of which is controlled by Japanese Pharmacopeia XIII. Oren-gedoku-to was prepared as follows: Scutellariae Radix (Japanese name: Ogon, 3g), Coptidis Rhizoma (Oren, 2g), Gardeniae Fructus (Sanshishi, 2g), and Phellodendri Cortex (Obaku, 1.5g) were added to 500 ml of water and extracted at 100℃ for 40 min. The extract was evaporated, lyophilized, and then dissolved in distilled water just before use. Oren-gedoku-to was administered orally once a day according to each protocol.

Haptens: 2,4-dinitrofluorobenzene (DNFB) used for in vivo studies was purchased from Nacalai Tesque (Kyoto, Japan) and 2,4-dinitrobenzenesulfonate (DNBS) used for in vitro studies was purchased from Tokyo Kasei Kougyo Co., LTD. (Tokyo, Japan).

Contact hypersensitivity (CHS) response: The CHS response to DNFB was determined by mouse ear-swelling test. Briefly, mice were epicutaneously sensitized on day 0 and 1 by application of 25 μl of 0.5% DNFB diluted in acetone-olive oil (4:1, v/v) onto shaved abdominal skin. Sensitized and unsensitized mice were challenged on day 6 by applying 10 μl of DNFB to each side of both ears. Ear thickness was measured using a dial thickness gauge (G-1A type, Peacock, Ozaki MFG., Co., LTD., Osaka, Japan) before and at appropriate times after the challenge. The results were expressed as average ear swelling (increase in ear thickness, μm) $\pm$ S.D. of six mice.

Reverse transcriptase-polymerase chain reaction (RT-PCR): Ear samples were collected from unsensitized, vehicle-treated or Oren-gedoku-to-treated mice (3 or 4 mice per group) and immediately homogenized with ISOGEN (Nippon Gene CO., LTD., Tokyo, Japan). Total RNA was extracted from ear samples according to

the manufacturer's instructions, and 2 μg RNA was reverse transcribed using oligo $(dT)_{12-18}$ primers and Superscript II RT (Life Technologies, France) for 50 min at 42℃. RNA detection was normalized using the housekeeping gene β-actin as a standard. The cDNA was then amplified using different sets of primers, including for β-actin (5'-primer: 5' -TGG AAT CCT GTG GCA TCC ATG AAA C-3'; 3'-primer: 5'-TAA A AC GCA GCT CAG TAA CAG TCC G- 3'), for CD8 (5'-primer: 5'-CAT TGA ATG TGA AGC CAG AGG-3'; 3'-primer: 5'-AGA AGC AGG ATG CAG ACT ACC-3'), for IFN-γ (5'-primer: 5'-GGT GAC ATG AAA ATC CTG CAG AGC-3'; 3'-primer: 5'-CGC TGG ACC TGT GGG TTG TTG ACC-3'), and TNF-α. (5'-primer: 5'-CCA CGC TCT TCT GTC TAC TG-3'; 3'-primer: 5'-GAA CCT GGG AGT AGA CAA GG-3'). The amplifications were conducted with 28 cycles for β-actin and 34 cycles for IFN-γ and for TNF-α and 36 cycles for CD8 (30 sec at 94℃, 30 sec at 60℃, 1 min at 72℃), and the PCR products were analyzed on 1.5% agarose gel.

Isolation of LNC: At the time of after or before challenge axillary and inguinal LNs were disrupted by gently rubbing between frosted ends of two glass microscopic slides. The cells were centrifuged at 400g for 5min and washed twice with phosphate buffer saline (PBS). The number of cells was counted by using Tülk solution (Wako Pure Chemical Industries, LTD., Osaka, Japan).

Flow cytometric analysis: On day 2 after challenge the cell suspension of skin-draining regional lymph nodes (LNs) were prepared as described above. The cell population of these cells was characterized by two-color flow cytometry. Briefly, $1x10^6$ cells were first preincubated with anti-CD16/32 (2.4G2) mAb to avoid non-specific binding of Abs to FcγR. Then the cells were incubated with a saturation amount of PE-conjugated anti-CD4 (H129-19) or anti-CD8 (53-6.7) mAb, and FITC-conjugated anti-CD3 (145-2C11) mAb. All staining reagents were obtained from BD Biosciences Pharmingen (San Diego, CA). After washing with PBS, the stained cells were analyzed by FACSCalibur (Becton Dickinson, San Jose, CA).

Hapten-specific lymphocyte proliferation in vitro: On day 5 after epicutaneous sensitization with DNFB LNCs were harvested as prepared above. The cells ($5x10^5$) were cocultured for 3 days at 37℃ in 96-well flat-bottom plates with mitomycin C-treated syngeneic spleen cells (10^6/well) from naive BALB/c mice, previously derivatized with DNBS as described.[17] Briefly, cells were incubated for 20 min at 37℃ with 4mM DNBS, pH8, in serum-free RPMI and washed in complete medium before use. The proliferative activity was assessed using a Cell Proliferation ELISA, BrdU (colorimetric) Kit (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's instructions. The results were expressed as absorbance (ABS) $\pm$ S.D., where ABS = (ABS in cultures of LNCs with DNBS-treated spleen cells) - (ABS in cultures of LNCs with untreated spleen cells).

Statistical analysis: Statistical comparison at each measurement time between groups was made by repeated measure ANOVA and unpaired t-test. Statistical analyses for other groups were performed by Kruskal-Wallis test followed by Mann-Whitney U test with Bonferroni correction.

Results

Effect of Oren-gedoku-to on DNFB induced CHS response

We first investigated the inhibitory effect of Oren-gedoku-to on the DNFB-induced CHS response. Oren-gedoku-to at a dose of 1g/kg was administered orally to mice once a day for 12 consecutive days after sensitization, and the challenge with DNFB was performed on day 6. Fig. 1 shows that continuous administration of Oren-gedoku-to significantly suppressed ear swelling as compared to the vehicle-treated group. Moreover, the duration of the ear swelling was shortened in Oren-gedoku-to-treated mice. These results clearly indicate that Oren-gedoku-to inhibited the DNFB-induced CHS response in mice.

We next examined the effect of the timing of Oren-gedoku-to administration on the suppression of the CHS response. Oren-gedoku-to was administered orally to mice once a day for 3 consecutive days on either days 0-2 or days 4-6 after sensitization. Administration of Oren-gedoku-to with either timing resulted in a significant reduction of ear swelling (Fig. 2). Thus, the administration of Oren-gedoku-to with different timings after sensitization or before the challenge was effective in inhibiting the CHS response.

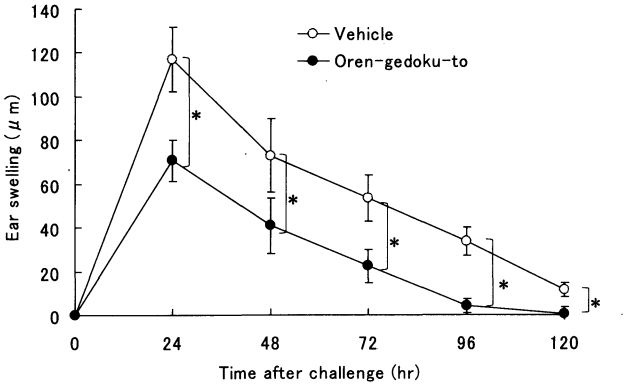

Fig. 1 Effect of Oren-gedoku-to on DNFB-induced CHS response. BALB/c mice were orally given vehicle (○) or 1g/kg Oren-gedoku-to (●) from days 0 to 11. On day 6, each ear was challenged on both sides with 0.2% DNFB. The thickness of the DNFB-challenged ears was measured and expressed as average ear swelling (μ m±S.D.) of six mice. *p<0.05

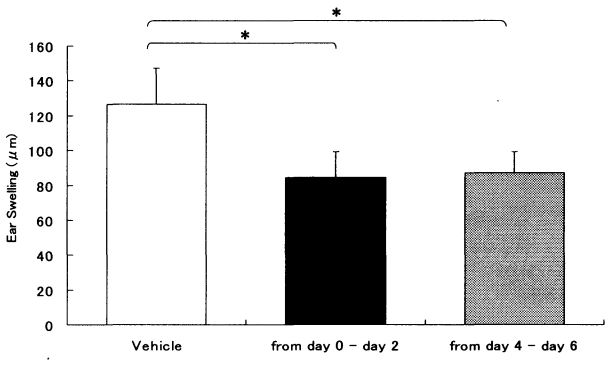

Fig. 2 The administration of Oren-gedoku-to at different timings was effective in inhibiting the CHS response. BALB/c mice were given vehicle, 1g/kg of Oren-gedoku-to on days 0 to 2 or on days 4 to 6 (after sensitization). The thickness of DNFB-challenged ears was measured at 24 h and expressed as average ear swelling (μ m±S.D.) of six mice. *p<0.05

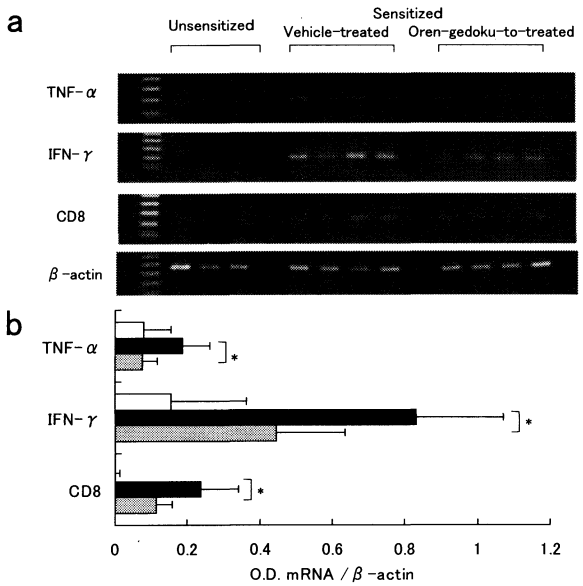

Fig. 3 Expressions of mRNA for CD8, IFN-γ and TNF-α in the ears of mice after the challenge with DNFB. CD8, IFN-γ and TNF-α mRNA expressions were analyzed using semiquantitative RT-PCR (a). BALB/c mice were orally given vehicle or Oren-gedoku-to from day 0 to 6, and then on day 6 each ear was challenged. Twenty-four hours after DNFB challenge mRNA was obtained from the ears of vehicle-treated or Oren-gedoku-to-treated mice. The ears from unsensitized mice were used as control. The results represent the ratio of each group to that to β-actin mRNA as standard densitometrically (b). Unsensitized (□), Control (■) or Oren-gedoku-to-treated (▒). Data are the mean ±S.D. of three or four mice. *p<0.05

Expressions of mRNA for CD8, IFN-γ and TNF-α in the ear of mice after challenge with DNFB

Since CD8$^+$ T cells, IFN-γ and TNF-α have been reported to be associated with the induction of the CHS response,[11,12] we investigated the effect of Oren-gedoku-to on the mRNA expressions of CD8, IFN-γ and TNF-α in ear tissue of mice 24 h after DNFB challenge by the use of RT-PCR. Oren-gedoku-to was administered orally to mice on days 0-6 after sensitization. Corresponding to the induction of the CHS response, the expressions of mRNA for CD8, IFN-γ and TNF-α were obviously increased in the ear of vehicle-treated and sensitized mice as compared with unsensitized normal mice, but they were substantially decreased in Oren-gedoku-to-treated mice (Fig. 3). These results demonstrated that the oral administration of Oren-gedoku-to markedly reduced the expressions of CD8, IFN-γ and TNF-α mRNA in ear tissue.

Lymphocyte population in draining LN of sensitized mice after DNFB challenge

The results in Fig. 3 demonstrate that the administration of Oren-gedoku-to to sensitized mice may diminish the infiltration of CD8$^+$ T cells into the challenged ear. Therefore, we investigated both the number and the population of lymphocytes in draining regional LNs isolated from treated mice on day 2 after the DNFB challenge. As shown in Fig. 4, the number of whole LNCs, CD8$^+$ T cells and CD4$^+$ T cells was markedly increased in sensitized mice after the challenge as compared with unsensitized mice. The administration of Oren-gedoku-to suppressed the increase in the number of these cells, but did not affect the ratio of CD8$^+$/CD4$^+$ T cells. These results suggest that the oral administration of Oren-gedoku-to can have a favorably suppressive effect on the increasing numbers of whole LNCs, CD8$^+$ T cells and

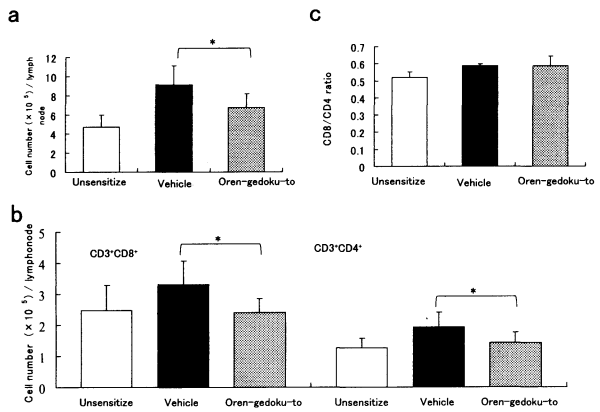

Fig. 4 Lymphocyte population in draining LNs of the sensitized mice after DNFB challenge. BALB/c mice were treated as described in Fig. 3, and then on day 6 each ear was challenged. Forty-eight hours after DNFB challenge the number of LNCs was counted (a). The numbers of $CD8^+$ and $CD3^+$ cells and $CD4^+$ and $CD3^+$ cells was calculated by multiplying the percentage of double positive cells, as determined by flow cytometry analysis, by the total number of cells recovered from each axillary and inguinal LNs (b). The ratio of $CD8^+$ T cells to $CD4^+$ T cells was calculated (c). Data are the mean $\pm$S.D. of six mice. *$p<0.05$

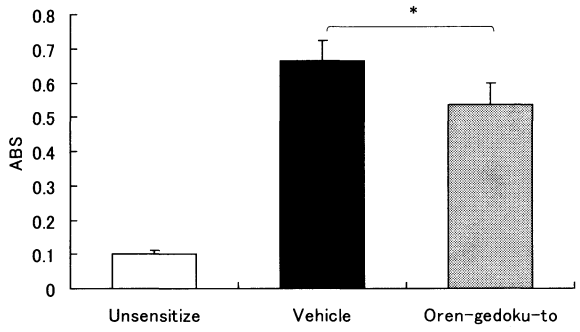

Fig. 5 Impaired development of hapten-specific LNs cells in Oren-gedoku-to-treated mice. LNCs harvested from vehicle-treated or Oren-gedoku-to-treated mice, on day 5 after epicutaneous sensitization, were restimulated in vitro for 3 days with 10^6 syngeneic mitomycin C-treated spleen cells with or without DNFB. LNCs proliferation was determined by BrdU Cell Proliferation ELISA kit. Results are expressed as $\triangle$ABS values (*i.e.*, ABS in culture lymphocytes with hapten-treated spleen cells - ABS from culture lymphocytes with untreated spleen cells.) Data are the mean $\pm$S.D. of six mice. *$p<0.05$

$CD4^+$ T cells in skin-draining regional LNs without affecting the $CD8^+/CD4^+$ T cell ratios.

Administration of Oren-gedoku-to inhibits hapten-specific proliferation of lymphocytes in vitro

We next examined the effect of Oren-gedoku-to on hapten-specific proliferation of whole LNC on day 5 after sensitization with DNFB. Oren-gedoku-to was administered orally to mice on day 0-5 after the sensitization. LNCs from vehicle-treated mice proliferated after *in vitro* re-stimulation with syngeneic haptenated cells. Administration of Oren-gedoku-to resulted in a significant inhibition of the hapten-specific proliferative response (Fig. 5).

Discussion

The immunopharmacological effect of Oren-gedoku-to on the hapten-specific CHS response in mice was demonstrated in this study. The administration of Oren-gedoku-to both decreased the magnitude of ear swelling in the CHS response and shortened the duration of swelling (Fig. 1). The inhibitory effect on ear swelling was observed even when Oren-gedoku-to was given orally at different timing schedules (Fig. 2). RT-PCR analysis revealed that the treatment with Oren-gedoku-to resulted in a marked decrease of mRNA expressions of CD8, IFN-γ and TNF-α in the challenged ear (Fig. 3). Oren-gedoku-to elicited impairment of the hapten-specific development of lymphocytes followed by a reduction in the number of skin-draining regional LNCs without affecting the ratio of $CD8^+$/ $CD4^+$ T cells (Fig. 4 and 5).

Clinically, Oren-gedoku-to has often been used for patients with eczema or pruritus due to relative Yang-excess of the middle or exterior of the body in the therapy of AD.[16] It has also been reported that Oren-gedoku-to exerts a suppressive effect on inflammatory bowel disease,[7] acetic acid-induced inflammation,[8] carrageenin cotton pellet-induced granuloma,[9] uveitis,[10] and the PC-induced CHS response.[11,12] Thus, Oren-gedoku-to has shown an anti-inflammatory effect in several models involving the CHS response. Nose *et al.* have reported that Oren-gedoku-to inhibited the PC-induced CHS response, and the inhibitory effect on ear swelling was shown when Oren-gedoku-to was given orally either after sensitization or before measurement.[12] Similarly, the present study indicated that the administration of Oren-gedoku-to at different timing points after sensitization inhibited ear swelling of DNFB-induced CHS (Fig. 2) and also shortened the affected period (Fig. 1).

CHS is recognized as a T cell-mediated cutaneous inflammatory reaction to haptens.[18] Epidermal Langerhans cells (LCs) play a pivotal role in the CHS response. LCs can either bind through MHC molecules on their surface directly to hapten or process the allergen internally into a complete Ag. LCs then migrate via afferent lymphatic

vessels into skin-draining regional LNs to present the haptenated peptide to naive T cells. As a result, T cells become activated and polarized toward a type 1 T cell profile. Then activated T cells down-regulate the expression of LN homing receptor (L-selectin) while increasing the expression and activity of adhesion molecules such as LFA-1 and VLA-4.[19,20,21] Upon challenging the skin with the same hapten, vascular endothelial cells expressed endothelial cell adhesion molecules including ICAM-1 and VCAM-1.[22,23] The interaction of VLA-4 and LFA-1 on the lymphocyte surface with endothelial cell adhesion molecules and extracellular matrix proteins facilitate the migration of immune T cells to the site of antigenic challenge.[24] The haptenated protein is presented by LCs and/or other APCs to the migrated T cells, which induce the production of type 1 cytokines such as IFN-γ and IL-2, thereby initiating the cutaneous inflammatory reaction.[18]

In the present study, the expressions of CD8, IFN-γ and TNF-α mRNA in the challenged ear were suppressed by the treatment with Oren-gedoku-to. It was previously reported that the skin inflammatory response to DNFB in sensitized mice is initiated by IFN-producing $CD8^+$ T cells, which migrate to the skin by 6 h after the challenge, and this is followed by recruitment of inflammatory cells including both $CD8^+$ and $CD4^+$ T cells 24-48 h after the challenge.[17] These results indicate that the inhibition of ear swelling by Oren-gedoku-to was correlated with reduction of $CD8^+$ T cells recruited in the skin challenged with DNFB. Possible reasons for the decrease in the number of $CD8^+$ T cells at the challenged site by Oren-gedoku-to would include: 1) impaired development of specific $CD8^+$ effector cells in LNs and 2) inability of effector cells to migrate to the site of inflammation. Since the number of $CD8^+$ T cells was decreased in skin-draining LNs from Oren-gedoku-to-treated mice after DNFB challenge, the former seems to be a plausible reason for the reduction of $CD8^+$ T cell number at the challenged site.

As was already mentioned above, the suppression of T cell activation may result in a decrease of the expressions of LFA-1 and VLA-4,[19,20,21] which are important molecules for the migration of lymphocytes to the challenged site in the CHS response.[24] Based on these and previous data, we speculate that Oren-gedoku-to also abrogated T cell migration due to the suppression of their activation.

In conclusion, the present study demonstrated that the inhibitory effect of Oren-gedoku-to on CHS reaction may be partly associated with the suppression of DNFB-specific lymphoproliferation followed by a reduction in the number of hapten-specific LNCs. Since LCs and T cells play a pivotal role for lymphocytes on hapten-specific proliferation in the CHS response,[18] Oren-gedoku-to seems to exert a suppressive effect on the proliferation of these cells. However, it remains unclear whether the efficacy of Oren-gedoku-to appears through LCs or through T cells, or possibly both. Further study will be needed to examine the inhibitory mechanism of Oren-gedoku-to in greater detail.

和文抄録

接触過敏反応 (CHS) に対する黄連解毒湯の抑制効果について検討した。1g/kg の黄連解毒湯を感作日より連続投与することで，DNFB 塗布による耳介の腫脹は軽減し，その持続時間も短縮した。また，黄連解毒湯の投与期間を変更（感作後 0–2 日間あるいは 4–6 日間の投与）しても抑制効果が認められた。耳介局所では，黄連解毒湯の連続投与により，CD8, IFN-γ および TNF-α の mRNA 発現は減弱した。所属リンパ節では，全リンパ節細胞，$CD8^+$ T 細胞，$CD4^+$ T 細胞の数が減少したが，CD8/CD4 比に変化はみられなかった。さらに，リンパ節細胞のハプテン特異的な増殖能は抑制された。以上の結果より，黄連解毒湯の CHS の抑制効果にハプテン特異的リンパ球の増殖抑制が関与していると考えられた。

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