A Kampo Formulation: Byakko-ka-ninjin-to (Bai-Hu-Jia-Ren-Sheng-Tang) Inhibits IgE-Mediated Triphasic Skin Reaction in Mice: The Role of Its Constituents in Expression of the Efficacy

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We have demonstrated that oral administration of a Kampo formulation, Byakko-ka-ninjin-to (Bai-Hu-Jia-Ren-Sheng-Tang), inhibited IgE-mediated triphasic skin reaction, including immediate phase response (IPR), late phase response (LPR) and very late phase response (vLPR), in passively sensitized mice with anti-DNP IgE antibody. Variant formulations of Byakko-ka-ninjin-to without Gypsum Fibrosum (Sekko), Glycyrrhiza Radix (Kan佐) or Oryzae Semen (Kobei) attenuated the inhibitory effect as compared with that of Byakko-ka-ninjin-to. The decreased effect of Byakko-ka-ninjin-to without Kan佐 was restored by the addition of Kan佐 to the variant formulations before oral administration, while the decreased effect of Byakko-ka-ninjin-to without Sekko could not be recovered by the addition of Sekko. Comparison of HPLC profiles of variant formulations without one crude drug with that of original Byakko-ka-ninjin-to revealed that some peaks could be detected only when five constituent crude drugs were simultaneously present during the preparation of Byakko-ka-ninjin-to formulation. Since elimination of Sekko from the Byakko-ka-ninjin-to constituents attenuated the efficacy although it did not show any activity per se, mutual interaction of Sekko with other constituents during the preparation may result in the production of new components. These findings suggest that the effect of Byakko-ka-ninjin-to formulation on cutaneous inflammatory disease can differ from the sum of the effect of the individual constituents.

Key words vLPR; Byakko-ka-ninjin-to; allergic inflammation; triphasic cutaneous reaction; Glycyrrhiza Radix; Gypsum Fibrosum

Previous studies have reported that mice passively sensitized with anti-dinitrophenol (DNP) IgE antibody exhibited IgE-mediated cutaneous reaction with an immediate phase response (IPR) at 1 h and a late phase response (LPR) at 24 h after the challenge of dinitrofluorobenzene (DNFB).¹¹—⁴ We recently found that the third-phase inflammatory reaction with intense and persisting infiltration of eosinophils, called “very late phase response” (vLPR), was induced following IPR and LPR in response to DNFB in actively and passively sensitized mice.⁵ The vLPR in the triphasic cutaneous reaction peaked at 8 days after the challenge, and was mainly mediated by T cells and partially by mast cells.⁵ This response was slightly observed in non-sensitized mice. Since the accumulation of eosinophils in vLPR was observed more markedly than that of LPR at 24 h, vLPR may be an important reaction in allergic inflammatory diseases.

We previously reported that some Kampo formulations and synthetic anti-allergic agents inhibited the IgE-mediated triphasic cutaneous reaction in this model.⁶—¹⁰ 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.⁸,¹⁰ 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. +/+/+ group that showed inhibitory effects against the triphasic response), similar to the effect of prednisolone as a positive control.⁹ 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,¹⁰ Kampo formulations in the +/+/+ and/or −/+/+ groups are expected to show such anti-PAF and LTB4 activities.¹⁰

In the present study, we investigated the effect of Byakko-ka-ninjin-to on murine IgE-mediated triphasic skin reaction in passively sensitized mice. We also examined the role of the constituents of Byakko-ka-ninjin-to in the expression of the efficacy against triphasic skin reaction, especially vLPR, which is believed to be associated with severe and chronic allergic reactions.

MATERIALS AND METHODS

Mice Specific pathogen-free BALB/c mice (6 weeks old) 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. 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.

Antigen and Chemicals Byakko-ka-ninjin-to (Bai-Hu-Jia-Ren-Sheng-Tang; TJ-34, lot. no. 24009040) is composed of five crude drugs (Table 1), of which the quality is controlled by Japanese Pharmacopoeia XIII. Byakko-ka-ninjin-to and its variant formulations were obtained from Tsumura and Co., Tokyo, Japan. Byakko-ka-ninjin-to was prepared as follows: a mixture of Glycyrrhiza Radix (Japanese name; Kan佐, 2 g), Oryzae Semen (Kobei, 8 g), Gypsum Fibrosum (Sekko, 15 g), Anemarrhenae Rhizoma (Chimo, 5 g) and Ginseng Radix (Ninjin, 1.5 g) was added to 500 ml of water

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and extracted at 100 °C for 40 min. The extract was evaporated and lyophilized. The five variant formulations of Byakko-ka-ninjin-to were prepared by removing one crude drug from each of the five constituents, i.e., formulations lacking one crude drug. The extract of each crude drug in the Byakko-ka-ninjin-to formulation was prepared by boiling in water for 50 min and freeze-dried to powder. Each formulation and extract was dissolved in distilled water and administered orally 2 h before and 2–6 d after antigen challenge.

HPLC pattern analysis, the so-called “fingerprint” method, was performed to assess the homogeneity of the formulation and to prepare batches of constant formulation, as described previously. Figure 1 shows the HPLC profile of Byakko-ka-ninjin-to and UV Spectra.

**Table 1. Botanical Origin of Constituents of Byakko-ka-ninjin-to**

<table>
<thead>
<tr>
<th>Crude drug (Japanese name)</th>
<th>Botanical origin* (Family name)</th>
<th>Ratio (g)</th>
<th>Specimen no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycyrrhizae Radix (Kanzo)</td>
<td>Glycyrrhiza uralensis FISCHER (Leguminosae)</td>
<td>2.0</td>
<td>15063531</td>
</tr>
<tr>
<td>Gypsum Fibrosum (Sekko)</td>
<td>Natural calcium sulfate CaSO₄·2H₂O</td>
<td>15.0</td>
<td>16030951</td>
</tr>
<tr>
<td>Oryzae Semen (Kobei)</td>
<td>Oryza sativa Linné (Graminaceae)</td>
<td>8.0</td>
<td>1706831</td>
</tr>
<tr>
<td>Anemarrhenae Rhizoma (Chimo)</td>
<td>Anemarrhena asphodeloides Bunge (Liliaceae)</td>
<td>5.0</td>
<td>17022501</td>
</tr>
<tr>
<td>Ginseng Radix (Ninjin)</td>
<td>Panax ginseng C. A. MAYER (Araliaceae)</td>
<td>1.5</td>
<td>1506701</td>
</tr>
</tbody>
</table>

* The botanical origins are based on Japanese Pharmacopeia XIII.

**Fig. 1. HPLC Profile of Byakko-ka-ninjin-to and UV Spectra**

A dosage of Byakko-ka-ninjin-to was extracted with H₂O–EtOH (9 : 1, 200 ml), filtered and analyzed by HPLC (HP-1090, Hewlett-Packard) under the following conditions: column, TSK gel 80 T S ODS (4.6 × 250 mm); mobile phase, 10 mM phosphoric acid: CH₃CN (linear gradient, 95 : 5 → 20 : 80, for 1 h); flow rate, 0.8 ml/min; oven temperature, 40 °C; injection volume, 5 μl.

A: HPLC pattern analyzed by absorbance at 220 nm. B: Contour plot of HPLC pattern by UV absorbance (190—420 nm). C: UV spectra of main peaks. Origins of peaks a, Anemarrhenae Rhizoma (mangiferin-7-O-glucoside); b, Glycyrrhizae Radix; c, Anemarrhenae Rhizoma (mangiferin); d, Anemarrhenae Rhizoma (isomangiferin); e, Glycyrrhizae Radix (apioliquiritin); f, Glycyrrhizae Radix (liquiritin); g, Unknown; h—k, Glycyrrhizae Radix; l, Glycyrrhizae Radix (glycyrrhizin).

DNFB was purchased from Nacalai Tesque, Kyoto, Japan, and dissolved in 100% ethanol. Prednisolone 21-acetate was purchased from Sigma Chemical Co., St. Louis, MO. It was suspended in 0.5% methylcellulose solution, and administered intraperitoneally 2 h before and 4—6 d after the challenge.

**Anti-DNP IgE Preparation** An anti-DNP monoclonal antibody (mAb)-producing cell line (EC1) was cultured in 10 ml of an equal volume mixture of RPMI-1640 and Dulbecco’s modified Eagle minimum essential medium with high glucose supplemented with 10% heat-inactivated fetal bovine serum (Gibco Laboratories, Life Technologies, Inc., Grand Island, NY) and 2 mM glutamine until it reached a confluent state. The supernatant was harvested, centrifuged at 400 × g, and stored at −80 °C until use. The IgE antibody titer was estimated to be 1 : 1024 by heterologous passive cutaneous anaphylaxis in rats injected intravenously with DNP-bovine serum albumin as an antigen.

**Induction of Skin Reaction in Mouse Ears** BALB/c mice were given an i.v. injection of a 1-ml aliquot of anti-DNP IgE mAb-containing fluid 24 h before DNFB challenge. Skin reaction was elicited by applying 10 μl of 0.1% DNFB...
in 100% ethanol to each side of each ear of sensitized mice. The reaction to DNFB was evaluated by measuring ear thickness using a dial thickness gauge (G-1A type, Peacock, Ozaki MFG., Co., Ltd., Osaka, Japan) immediately before and at appropriate times after the challenge. The results were expressed as average ear swelling (increase in ear thickness, μm) ± S.D. of 3 mice.

**Statistical Analysis** Statistical significance of difference between the groups was determined by using Steel's test for the ear swelling experiments.

**RESULTS**

**Effect of Byakko-ka-ninjin-to and Its Variant Formulations on Triphasic Skin Reaction in Passively Sensitized Mice** Oral administration of Byakko-ka-ninjin-to inhibited IPR, LPR and vLPR in a dose-dependent manner (Fig. 2). Prednisolone was also effective at inhibiting triphasic skin reaction. Thus, Byakko-ka-ninjin-to as well as prednisolone belong to the 1/1/1 group according to the efficacies for IPR/LPR/vLPR.

To investigate the role of the crude drugs composing the formulation in expression of the efficacy, we next examined the anti-allergic effect of five variant formulations of Byakko-ka-ninjin-to (Fig. 3). None of these variant formulations, Byakko-ka-ninjin-to without Kanzo, Sekko, Kobei, Chimo or Ninjin, showed any inhibitory effect against IPR, as compared with the original Byakko-ka-ninjin-to. Three of the variant formulations, those without Kanzo, Sekko, or Kobei, markedly reduced the inhibitory effect on LPR and vLPR on a yield basis of Byakko-ka-ninjin-to to the untreated control level. In contrast, the two other variant formu-
lations, those without Chimo or Ninjin, significantly inhibited vLPR, but their effects were less active than the original.

Effect of Individual Crude Drugs of Byakko-ka-ninjin-to on Triphasic Skin Reaction in Passively Sensitized Mice

The above results indicated that Kanzo, Sekko and Kobei were primarily involved in the development of Byakko-ka-ninjin-to-mediated inhibition of skin reaction, because elimination of the crude drugs from the formulation decreased or abrogated the efficacy. We therefore investigated the effect of the five crude drugs in the formulation on triphasic skin reaction, especially vLPR (Fig. 4). Kanbo and Kebi extracts were significantly effective at inhibiting vLPR, while the other crude drugs did not show any discernible inhibitory effect. In particular, Sekko did not affect the inhibition of skin reaction at any dose used.

Role of the Constituents in Byakko-ka-ninjin-to Formulation in Expression of the Efficacy

To further determine the significance of the constituents of Byakko-ka-ninjin-to formulation for expression of the efficacy, we investigated the anti-allergic effect of reconstituted formulations of Byakko-ka-ninjin-to, e.g. Byakko-ka-ninjin-to without Kanbo+Kanbo extract, as compared with original Byakko-ka-ninjin-to. As shown in Fig. 5, the diminished efficacy of Byakko-ka-ninjin-to without Kanbo on vLPR was apparently restored by the addition of Kanbo extract to the variant formulation before oral administration. Kanbo at the dose estimated in yield showed a significant inhibition of the skin reaction. This lowered anti-allergic effect of Byakko-ka-ninjin-to without Sekko could not be improved, however by the reconstitution of Sekko to the variant formulation before the administration (Fig. 6). These results indicate that elimination of Sekko from Byakko-ka-ninjin-to constituents before preparing the formulation resulted in a marked decrease of the efficacy in comparison with original Byakko-ka-ninjin-to, although Sekko itself was not active. This result was seem-

Fig. 4. Effect of Byakko-ka-ninjin-to Constituents on Triphasic Skin Reaction in Passively Sensitized Mice

Each crude drug in Byakko-ka-ninjin-to was given orally 2 h before and 2 to 6 d after DNFb challenge. Prednisolone was given intraperitoneally 2 h before and 4 to 6 d after the challenge. Each value represents mean±S.D. of 3 mice. *p<0.05, **p<0.01 by Steel's test.

Fig. 5. Effect of a Mixture of Byakko-ka-ninjin-to without Kanbo and Kanbo Extract on Triphasic Skin Reaction in Passively Sensitized Mice

Byakko-ka-ninjin-to, Byakko-ka-ninjin-to without Kanbo, Kanbo extract, or a mixture of Byakko-ka-ninjin-to without Kanbo and Kanbo extract were given orally 2 h before and 2 to 6 d after DNFb challenge. Prednisolone was given intraperitoneally 2 h before and 4 to 6 d after the challenge. Each value represents mean±S.D. of 3 mice. *p<0.05, **p<0.01 by Steel's test.

Fig. 6. Effect of a Mixture of Byakko-ka-ninjin-to without Sekko and Sekko on Triphasic Skin Reaction in Passively Sensitized Mice

Byakko-ka-ninjin-to, (Byakko-ka-ninjin-to without Sekko), Sekko, or a mixture of (Byakko-ka-ninjin-to without Sekko) and Sekko were given orally 2 h before and 2 to 6 d after DNFb challenge. Prednisolone was given intraperitoneally 2 h before and 4 to 6 d after the challenge. Each value represents mean±S.D. of 3 mice. *p<0.05, **p<0.01 by Steel's test.
ingly quite different from that of a mixture of Byakko-ka-ninjin-to without Kanzo and Kanzo extract.

HPLC Profiles of the Variant Formulations of Byakko-ka-ninjin-to

Figure 7 shows HPLC profiles of the variant formulations by fingerprint analysis. The contour plot of UV absorbance intensity of the compounds shows all of the compounds that have detectable UV absorbance in the extracts from the formulations. The six peaks a, c, d, e, f and l of the formulations were identified by comparison with retention time and with the UV spectra of standard compounds. The profiles of five variant formulations were apparently different from that of Byakko-ka-ninjin-to formulation (Figs. 1 and 7). For example, peaks b and e—l in the profile of Byakko-ka-ninjin-to disappeared when Kanzo was eliminated from the formulation. Since Byakko-ka-ninjin-to without Kanzo showed decreased efficacy and Kanzo by itself was active at inhibiting skin reaction (Figs. 3—5), the peaks that disappeared may be important for expression of the activity. Byakko-ka-ninjin-to without Chimo and without Ninjin showed the disappearance of peaks a, c, g, j and k, or peaks g, j and k, respectively, and less inhibitory effects on the skin reaction than Byakko-ka-ninjin-to. In contrast, in Byakko-ka-ninjin-to without Sekko peaks g, j and k had disappeared and there was a dramatic decrease in efficacy. (Figs. 3—5). The pH of Byakko-ka-ninjin-to without Sekko is approximately 5.5, and is almost the same as that of the original Byakko-ka-ninjin-to (data not shown).

Although all variant formulations without one crude drug showed HPLC profiles commonly lacking peaks g, j and k, the inhibitory effects on vLPR varied from case to case. The results are summarized in Table 2.

DISCUSSION

We recently found that passive sensitization with anti-DNP
IgE antibody followed by the challenge of DNFB to the mouse ear can induce triphasic cutaneous reactions (ear swelling) of IPR, LPR and vLPR peaking at 1 h, 24 h and 8 d after the antigen challenge, respectively. IPR was absent in mast cell-deficient mice but LPR was observed, and vLPR was partly attenuated. LPR is a T cell-independent response, while vLPR is almost completely absent in T cell-deficient nude mice. Thus, a third-phase response (vLPR) with massive eosinophilic infiltration actually represents an important inflammatory reaction mediated by T cells and partially by mast cells. Byakko-ka-ninjin-to has recently been shown to possess an inhibitory effect on biphasic skin reaction by interfering with the cutaneous response caused by histamine and tumor necrosis factor (TNF)-α, and on contact dermatitis through the inhibition of interferon (IFN)-γ production. The present study demonstrated that the oral administration of Byakko-ka-ninjin-to inhibited IgE-mediated triphasic skin reaction in a dose-dependent manner (Fig. 2). This finding seems to reflect the beneficial effect of the oral use of Byakko-ka-ninjin-to on atopic dermatitis of dry and warm skin.

We also focused our attention on vLPR to determine the role of the constituents of Byakko-ka-ninjin-to in the development of the anti-allergic effect. From the results using the crude drugs composed of Byakko-ka-ninjin-to and the variant formulations (Figs. 3—6), elimination of Kanzo from the formulation resulted in a marked decrease of the effect. However, the decreased activity of Byakko-ka-ninjin-to without Kanzo was recovered by the addition of Kanzo to the formulation before the administration, because Kanzo itself was active. Disappearance of the peaks in the HPLC profile of Byakko-ka-ninjin-to without Kanzo may, in part, be due to its absence (peaks b, e, f, g, h—k, and l).

Elimination of Sekko (CaSO₄·2H₂O) from the formulation, on the other hand, also showed a marked reduction in the effect, although the active Kanzo is part of this variant formulation. The decreased effect of Byakko-ka-ninjin-to without Sekko could not be restored by its addition to the formulation, in contrast to the case of Byakko-ka-ninjin-to without Kanzo. Sekko may play an important role in the preparation process of Byakko-ka-ninjin-to to obtain an effective formulation containing some peaks resulting from the mutual interaction of Sekko with other crude drugs, but it did not directly inhibit per se, in contrast to Kanzo. These results may be associated with the clinical observations that Byakko-ka-ninjin-to containing increasing doses of Sekko has actually become successively more effective in terms of the holistic patterns of symptoms and individual pathogenic alterations, the so-called “SHO”, of patients with atopic dermatitis.

Byakko-ka-ninjin-to without Kobei or without Ninjin showed similar HPLC patterns to Byakko-ka-ninjin-to without Sekko (Fig. 7). Byakko-ka-ninjin-to without Ninjin was still active in inhibiting skin reaction, although a slight reduction of its anti-allergic effect was observed (Fig. 3). In contrast, without Sekko it had a markedly reduced effect, suggesting that other components in the Byakko-ka-ninjin-to without Ninjin, which can’t be detected by HPLC analysis, such as polysaccharides and peptides, are responsible for expression of the efficacy. Since Kobei was effective at inhibiting skin reaction (Fig. 4), however, the marked decrease of the inhibitory effect by Byakko-ka-ninjin-to without Kobei may be due to the absence of the active components of Kobei. The peaks g, j and k could not be commonly found in the profiles of any of the variant formulations, including Byakko-ka-ninjin-to without Sekko. Therefore, these peaks may appear only when the five crude drugs are simultaneously present during the preparation process. Further chemical study will be needed to examine this issue in detail.

In conclusion, we demonstrated that Byakko-ka-ninjin-to was effective at inhibiting IgE-mediated triphasic cutaneous reaction in mice. Three constituent crude drugs, Kanzo, Sekko and Kobei, were primarily responsible for the expression of the Byakko-ka-ninjin-to-induced efficacy. Sekko may contribute to the expression of the effect of Byakko-ka-ninjin-to through its interaction with other crude drugs during the decoction. Some peaks in the HPLC profile of Byakko-ka-ninjin-to were detected only when the five constituent crude drugs were simultaneously present during the preparation. This suggests that the effect of Byakko-ka-ninjin-to on cutaneous inflammatory disease may differ from the sum of the effects of the individual constituents.

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