

Effect of Bakumijiogan, an Herbal Formula in Traditional Chinese Medicine, on Atopic Dermatitis-Like Skin Lesions Induced by Mite Antigen in NC/Jic Mice

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We evaluated the effectiveness of bakumijiogan (BJG), an herbal formula in traditional Chinese medicine used to treat atopic dermatitis (AD), using a NC/Jic mouse model of AD. AD symptoms were induced by repeated injections of *Dermatophagoides farinae* antigen (Df-antigen) into the ear auricle at 2- to 3-d intervals for 16 d. Ear thickness dramatically increased up to 16 d after the first injection of Df-antigen. Daily oral administration of BJG from 7 d before to 16 d after the first injection significantly reduced ear swelling. Serum concentrations of total immunoglobulin (Ig)E and Df-antigen-specific IgG₁ were augmented when assayed 17 d after the first injection of Df-antigen, and these increases were slightly suppressed by BJG administration. Serum interferon (IFN)- γ and lesional IFN- γ mRNA levels were significantly higher, whereas lesional IL-1 α and tumor necrosis factor- α mRNA levels were lower in BJG-treated mice than those in control mice. These results suggest that BJG suppressed AD-like symptoms by correcting the Th1/Th2 imbalance skewed toward Th2. Evaluation of herbal constituents in BJG revealed that the combination of two herbal ingredients, ophiopogon tuber and schisandra fruit, mainly contributed to the effects of BJG.

Key words atopic dermatitis; NC mouse; bakumijiogan; ophiopogon tuber; schisandra fruit

Atopic dermatitis (AD) is a common skin disease characterized by pruritus, eczematous skin lesions, and a chronically relapsing course.²⁾ The pathogenesis of AD is multifactorial, and genetically determined immunologic aberrations are thought to play an important role in it. The mechanisms involved in disease development are incompletely understood, but mite antigen is thought to be one of the pathogens because patients have elevated serum levels of immunoglobulin-E (IgE) against the allergen.³⁾ There is some evidence that T-cell responses to the allergen are crucial for AD aggravation.⁴⁾

It has been accepted that topical steroid therapy is crucial for AD management, but they cannot be used for long periods because of frequent side effects. Steroid therapy can improve topical cutaneous symptoms, but cannot completely cure the disease. Many patients therefore choose to explore complementary/alternative medicines including herbal medicine.⁵⁾ Traditional Chinese medicine (TCM) and traditional Japanese medicine (Kampo) have been receiving increasing attention as alternative sources of treatment for chronic diseases, including AD. TCM is believed to have the potential to treat patients holistically by supporting the patient's self-healing power.^{6,7)} However, the dearth of scientific evidence of TCM has restricted its popular use.

A murine strain, NC, is a well-known animal model for AD and has been extensively used to elucidate AD pathogenesis and development. NC mice spontaneously develop AD-like eczematous skin lesions with IgE hyperproduction when raised under conventional conditions, but not under specific pathogen-free conditions.⁸⁾ It was revealed that environmental antigens such as *Dermatophagoides farinae* (Df) were involved in the pathogenesis of AD-like symptoms in NC mice. Repeated extraneous injections of the antigen to NC mice caused severe and settled dermatitis, which could then be used as a model to evaluate new pharmaceuticals for AD

treatment.^{9–11)}

We evaluated the effect of bakumijiogan (BJG) on the AD model induced by repeated Df-antigen injections in NC/Jic mice. BJG is a modified herbal formula of rokumigan. Rokumigan consists of six crude drugs (Table 1) and is a basic formula used to treat *kidney-yin* deficiency, which according to the TCM theory is a condition characterized by tidal fever, spontaneous sweating, dizziness, tinnitus, spermatorrhea (in males), and sexual intercourse in dream (in females). Six crude drugs contained in rokumigan and two additional crude drugs, ophiopogon tuber (bakumondo in Japanese, B) and schisandra fruit (gomishi in Japanese, G), constitute BJG (Table 1). In the TCM theory, the targeted conditions for BLG use include deficiency of *lung-kidney-yin*, reversed flow of *qi*, tidal fever, and night sweats. BJG has therefore been given to patients with asthma, AD, or systemic lupus erythematosus.¹²⁾

MATERIALS AND METHODS

Preparation of Herbal Formulas All crude drugs in the Japanese Pharmacopoeia XV¹³⁾ were purchased from Tsumura Co. Ltd. (Tokyo). The composition of BJG and related formulas are shown in Table 1. Each dried herbal mixture of the formula was boiled in 20-times weight of distilled water for 60 min. Decoctions were lyophilized and the powder was maintained at room temperature under desiccated conditions until use. Yields of the extract from herbal medicines are shown in Table 1. To prepare the sample for the animal experiments, lyophilized powder was suspended in distilled water at appropriate proper concentrations. The dose of each herbal formula is shown in Table 1, which was equivalent to ten-fold value of the human dosage.

An HPLC fingerprint of BJG is shown in Fig. 1. Lyophilized powder of BJG (0.5 g) was extracted with

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Table 1. Components of Crude Drugs in Kampo Medicine

English name	Japanese name	Rokumigan	Bakumijiogan (BJG)	Rokumigan+B	Rokumigan+G	B+G
Rehmannia root	Jio	5 g ^{a)}	5 g	5 g	5 g	—
Cornus fruit	Sansyuyu	3 g	3 g	3 g	3 g	—
Discorea rhizome	Sanyaku	3 g	3 g	3 g	3 g	—
Alisma rhizome	Takusya	3 g	3 g	3 g	3 g	—
Poria sclerotium	Bukuryo	3 g	3 g	3 g	3 g	—
Moutan bark	Botampi	3 g	3 g	3 g	3 g	—
Ophiopogon tuber (B)	Bakumondo	—	3 g	3 g	—	3 g
Schisandra fruit (G)	Gomishi	—	2 g	—	3 g	3 g
Ratio of yield ^{b)}		25.5%	32.4%	32.8%	27.9%	38.0%
Dosage of the extract to mice ^{c)}		1.02 g/kg/d	1.62 g/kg/d	1.26 g/kg/d	1.09 g/kg/d	0.456 g/kg/d

a) Weight of each crude drug in a daily dosage. b) The decoctions were prepared according to Materials and Methods, and ratio of yield was calculated as % of dried weight of the decoction to the weight of original crude drug. c) Calculated as 10-fold of human daily dosage.

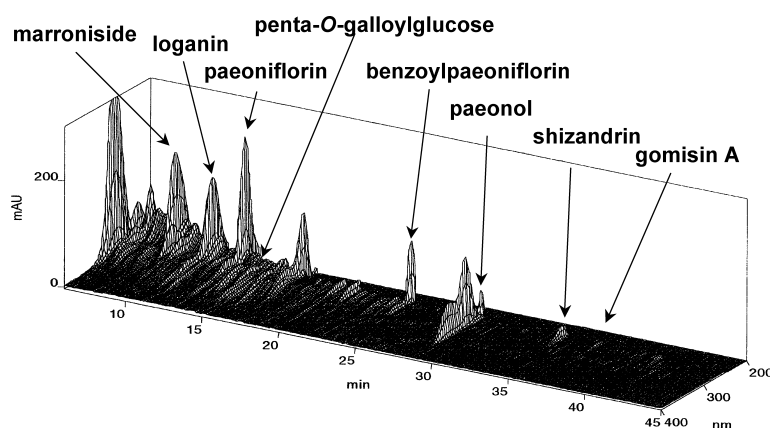


Fig. 1. Fingerprint Pattern of Bakumijiogan (BJG)

HPLC conditions were as follows: sample, 25 μ l of BJG solution (25 mg/ml in MeOH); column, TSK-GEL ODS-80_{TS} 4.6 $\times$ 250 mm, mobile phase, 0.05 M AcOH–AcONH₄ buffer (pH 3.6): CH₃CN 90:10 (0 min) $\rightarrow$ 0:100 (80 min), linear gradient; flow rate, 1.0 ml/min; column temperature, 40 $^{\circ}$ C; and detection, UV 200–400 nm. Compounds were identified by comparison of the retention times and UV spectra with authentic samples.

MeOH (10 ml) by sonication for 30 min, and the supernatant (25 μ l) was subjected to HPLC at the following conditions: system, Shimadzu LC-10A_{VP} (Kyoto); column, TSK-GEL ODS-80_{TS} (4.6 $\times$ 250 mm, Tosoh, Tokyo); mobile phase, 0.05 M AcOH–AcONH₄ buffer (pH 3.6)/CH₃CN 90:10 (0 min) $\rightarrow$ 0:100 (80 min), linear gradient; flow rate, 1.0 ml/min; column temperature, 40 $^{\circ}$ C; and detection, 200–400 nm by a photodiode array detector.

Animal Experiments Experimental procedures were approved by the Animal Care Committee at Graduate School of Pharmaceutical Sciences, Nagoya City University, Nagoya, in accordance with the guidelines of the Japanese Council on Animal Care.

Male NC/Jic mice (7–8 week old) were purchased from Clea Japan Inc. (Tokyo). They were housed in a temperature-controlled room (at 23 $\pm$ 1 $^{\circ}$ C) with lighting from 7 a.m. to 7 p.m. and freely access to food (CE-2, Oriental Yeast, Tokyo) and water under conventional conditions. Standardized mite *D. farinae* allergenic extract (1:100 (w/v), endotoxin-free) (Greer, Lenoir, NC, U.S.A.) was diluted to 1 mg/ml by saline, and cutaneously injected into the right ear (10 μ l/ear) on days 0 (first injection), 2, 4, 7, 9, 11, 14, and 16. Herbal extracts were orally administered daily from 7 d before to 16 d after the first injection. The normal group did not receive cutaneous injections, and were orally administered distilled water (10 ml/kg body weight). The vehicle group was treated with cutaneous injections of saline into the right ear and gavage of

distilled water. For the control group, distilled water was gavaged instead of herbal extracts every day. Ear thickness was measured using a dial thickness gage (Peacock model G-1A, Ozaki MFJ, Tokyo) 1 and 24 h after injection, and data were recorded as the difference from the values before the first injection. Mice were sacrificed by excessive anesthesia with ether on day 17. Serum, postauricular lymph node, and ear samples were collected.

Enzyme-Linked Immunosorbent Assay (ELISA) for Serum Immunoglobulins and Interferon (IFN)- γ Total serum IgE concentrations were measured by sandwich ELISA using 96-well assay plates (Sumitomo Bakelite, Tokyo). Wells were coated with purified anti-mouse IgE (2 μ g/ml, BD Biosciences, Franklin Lakes, NJ, U.S.A.). After blocking with a 0.5% solution of bovine serum albumin, sample or standard murine IgE (BD Biosciences) was added to wells. The amount of IgE bound to a well was indirectly assayed by using 1:1000 dilution of avidin-horseradish peroxidase (HRP) (BD Biosciences) via biotinylated anti-mouse IgE (0.5 μ g/ml, BD Biosciences). Concentration of total IgE was calculated by the calibration curve of standard mouse IgE.

Serum concentrations of Df-antigen-specific IgG₁ and IgG_{2a} were assayed by sandwich ELISA using a Falcon 96-well immunoplate (BD Biosciences). Wells were coated with Df-antigen solution (50 μ g/ml in 0.15 M phosphate-buffered saline, pH 7.2), and samples were detected with 1:20000 di-

Table 2. Primers Used and PCR Conditions for RT-PCR Analysis

	Sense primer	Anti-sense primer	Size of the products	Cycles	Annealing
IL-1 α	CTTCAAGGAGAGCCGGGTGA	GCAACACGGGCTGGTCTTCT	422 bp	37	55 °C
IL-4	TCAACCCCCAGCTAGTTGTCA	CATCGAAAAGCCGAAAGAG	313 bp	40	50 °C
IFN- γ	GCTACACACTGCATCTTGCTTTG	CACTCGGATGAGCTCATTGAATGC	407 bp	40	55 °C
GAPDH	TGTTCTACCCCCAATGTGTC	ATTATGGGGGTCTGGGATGGA	422 bp	30	55 °C

Table 3. Primers for Real Time RT-PCR Analysis

	Sense primer	Anti-sense primer
IL-1 α	TTCAAGGAGAGCCGGGTGA	TGCTGATCTGGGTGGATGG
IL-4	CAGCAACGAAGAACCACACAG	ATCGAAAAGCCGAAAGAGTC
TNF- α	ACACCGTCAGCCGATTTGC	TTTGAGCTCAGCCCCCTCA
GAPDH	CAAGATTGTCAGCAATGCATCC	CCTTCCACAATGCCAAAGTTG

luted solution of HRP-labeled anti-mouse IgG₁ (Bethyl Laboratories, Inc. Montgomery, TX, U.S.A.) or 1 : 1000 diluted solution of HRP-labeled anti-mouse IgG_{2a} (Bethyl). Data were expressed as the titer calculated by the standard murine anti-Df-antigen serum, which was prepared according to the method of Inagaki *et al.*¹⁴⁾ with slight modifications. Briefly, Df-antigen was suspended in a 10 mg/ml Al(OH)₃ solution (final concentration of Df-antigen, 100 μ g/ml), and 0.2 ml of this suspension was injected intraperitoneally into 4-week-old female Balb/c mice (Japan SLC, Hamamatsu) six times at 2-week intervals. Whole blood was collected 7d after the sixth injection.

Serum IFN- γ concentrations were measured using a commercial kit (Quantikine Mouse IFN- γ Immunoassay kit, R&D Systems, Minneapolis, MN, U.S.A.) according to the manufacturer's instructions. Detection limit was >0.5 pg/ml.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Total RNA was extracted from about 30 mg of pooled ear samples in each group using TRIzol reagent (Invitrogen, Carlsbad, CA, U.S.A.). DNase I (Promega, Madison, WI, U.S.A.)-treated RNA (500 ng) was reverse-transcribed using 100 unit of reverse transcriptase (ReverTra Ace, Toyobo, Osaka) with 0.1 mM oligo(dT) primer in a total volume of 20 μ l. An aliquot (2 μ l) of the RT mixture was used for PCR with 1 unit AmpliTaq Gold (Applied Biosystems, Foster City, CA, U.S.A.) and 10 μ M primers. Primer sequences are shown in Table 2. The amplification program consisted of one cycle at 94 °C for 10 min, and the cycles shown in Table 2 at 95 °C for 30 s, followed by annealing at various temperatures (Table 2) for 30 s and 72 °C for 30 s. PCR products were resolved on 1.5% agarose gels and visualized by ethidium bromide staining.

Quantitative RT-PCR Real-time RT-PCR was performed on an ABI PCR7300 System (Applied Biosystems) using two-fold diluted Power SYBR Green PCR Master Mix (Applied Biosystems). Primer sequences were obtained from an online library and are shown in Table 3. Δ Ct was determined by subtracting the mean cycle threshold (Ct) of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from that of the targeted cytokines. Data were expressed as the fold change to the level of control mice.

Statistical Analyses Statistical analyses were conducted by repeated one-way analysis of variance (ANOVA), com-

bined with Dunnet's multiplex comparison analysis. A probability value of less than 0.05 was considered statistically significant.

RESULTS

Intradermal injection of Df-antigen into the ears of NC/Jic mice (control group) resulted in significantly greater ear swelling one day after the first injection compared with vehicle-injected mice (vehicle group). The ear auricle in the control group presented with a reddish-colored wound and dried skin seven days after the first injection, and these symptoms were augmented daily. Compared with the normal group (not injected), the vehicle group showed slightly greater ear swelling 12 d post-injection. On day 17, the weight of the postauricular lymph node in the control group was significantly higher than that of the normal and vehicle groups (normal, 1.9 ± 0.5 mg; vehicle, 2.3 ± 0.8 mg; control, 6.8 ± 1.4 mg). Ear swelling of BJG-treated mice was lower than control over the experimental period of 17 d, and the suppression was statistically significant ($p < 0.01$) until day 7 (Fig. 2a). The area under the curve of ear swelling from days 0 to 17 was significantly reduced to 68% of that of the control group ($p < 0.05$) by BJG administration (Fig. 2b). Hypertrophy of the postauricular lymph node on day 17 was not significantly reduced by BJG administration (BJG group, 6.6 ± 0.9 mg).

Serum concentrations of total IgE, Df-specific IgG₁ and IgG_{2a} were measured on day 17. Control mice exhibited significantly higher serum concentrations of total IgE, Df-specific IgG₁ and IgG_{2a} compared with normal and vehicle groups. Serum concentrations of total IgE and Df-specific IgG₁ in BJG-treated mice were slightly lower than in control, whereas serum Df-specific IgG_{2a} in BJG-treated mice was slightly higher than in control (Table 4).

While Df-antigen injection did not affect the serum concentration of IFN- γ , it was significantly elevated in BJG-treated mice (Fig. 3a). IFN- γ mRNA expression in the ear was slightly induced by treatment with Df-antigen, and mRNA levels were further increased in BJG-treated mice (Fig. 3b). Changes in IFN- γ mRNA expression paralleled serum IFN- γ levels.

mRNA levels of the inflammatory cytokines IL-4, IL-1 α and tumor necrosis factor (TNF)- α in the ear of control

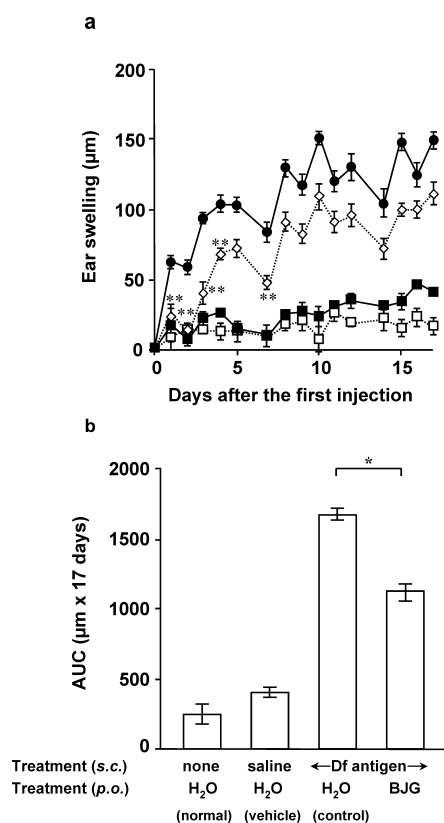


Fig. 2. Effect of Bakumijogan (BJG) on Ear Swelling in NC/Jic Mice Topically Injected with Df-Antigen

(a) NC/Jic mice were orally treated with BJJG from days -7 to 17 , and ear swelling was consecutively measured. Open square, normal group (no s.c. or oral treatment with H_2O); closed square, vehicle group (s.c. injection of saline and oral treatment with H_2O); closed circle, control group (s.c. injection of Df-antigen and oral treatment with H_2O); open diamond, BJJG group (s.c. injection of Df-antigen and oral treatment with BJJG, 1.62 g/kg/d). (b) Area under the curve (AUC) of Fig. 2a is shown, that was calculated by the trapezoid rule. Data are represented as mean $\pm$ S.E. ($n=7$). * $p<0.05$, ** $p<0.01$ vs. control group.

Table 4. Serum Concentrations of Immunoglobulins in NC/Jic Mice Topically Injected with Df-Antigen

	Total serum IgE (μ g/ml)	Df-specific IgG ₁ (titer $\times 10^{-2}$)	Df-specific IgG _{2a} (titer $\times 10^{-2}$)
Normal	0.475 ± 0.094	No detection	No detection
Vehicle	0.385 ± 0.110	No detection	No detection
Control	$1.95 \pm 0.30^\dagger$	2.56 ± 0.35	1.93 ± 0.35
BJG	1.29 ± 0.37	1.67 ± 0.20	3.13 ± 1.21

For murine groups, see the legend to Fig. 2. Data were represented as mean $\pm$ S.E. ($n=7$). $^\dagger p<0.01$ vs. vehicle group.

group were higher than those in the normal and vehicle groups. In BJJG-treated mice, IL-4 mRNA expression was slightly higher, whereas IL-1 α and TNF- α mRNA expressions were lower than those in the control group (Figs. 3b, c).

We identified the herbal ingredients contributing to the suppressive effect of BJJG on AD-like symptoms. We administered rokumigan, BJJG (rokumigan+B+G), rokumigan+B, rokumigan+G, and B+G (Table 1) to the mice. Ear swelling of NC mice administered rokumigan was temporarily lower than that in the control group on days 3 and 9 (data not shown), but total suppression of ear swelling was not observed. Neither rokumigan+B nor rokumigan+G showed significant suppression of ear swelling, whereas B+G signifi-

cantly suppressed the ear swelling of NC mice ($p<0.01$), and suppression was comparable with that caused by BJJG (Fig. 4).

DISCUSSION

We showed that BJJG significantly suppressed the development of AD-like symptoms induced by repeated injections of Df-antigen in NC mice. Df-antigen contains Der f1 as a major antigen, which inhibits endogenous protease inhibitors and disrupts the integrity of interepithelial tight junctions^{15,16} in order to degrade the dermal barrier function, resulting in the development of AD-like symptoms in NC mice. AD-like symptoms are characterized by severe ear swelling with a thickened epidermis, eosinophil infiltration, fibrosis of the dermis, and high IgE titer in serum, all of which resembled human AD.⁹

Most patients suffering from AD have a higher serum concentration of IgE, and Df-antigen-specific Th2 cells are detected in peripheral blood.¹⁷ The Th2 condition has the tendency to produce IL-4, histamine, and IgE.^{18,19} In the present NC mouse model, repeated injections of Df-antigen into the ear auricle resulted in IgE hyperproduction with upregulation of IL-4 and TNF- α mRNA levels at the lesion site. These changes in cytokine levels are similar to those of AD patients.^{20,21} BJJG prevented the increase of neither IL-4 mRNA levels at the lesion site nor serum IgE and Df-antigen-specific Ig levels. BJJG significantly increased serum IFN- γ levels, which is a Th1 cytokine. Although IL-4 expression in the ear was only slightly increased by BJJG treatment, IFN- γ expression notably increased in the ear, while IL-1 α and TNF- α expressions were slightly decreased. These results suggest that BJJG corrects the Th1/Th2 balance skewed toward Th2 by predominantly promoting systemic Th1 functions, which led to the improvement of ear swelling presumably through simultaneous decrease in IL-1 α and TNF- α at the lesion site.

Gao *et al.* examined the effects of four herbal formulas in Kampo or TCM, hochuekkito, jumentaihoto, shofusan and orangedokuto, on NC/Nga mouse dermatitis model by repeated painting of Df-antigen solution on the ears after tape stripping.²² All herbal formulas effectively suppressed dermatitis, and prevented increase of IL-4 mRNA expression and decrease of IFN- γ levels. Jumentaihoto and hochuekkito also inhibited IgE hyperproduction. The four herbal formulas previously reported and BJJG in the present investigation suppressed the AD-like symptoms induced by Df-antigen in NC mice through essentially the same mechanism, *i.e.*, correction of Th1/Th2 imbalance, although the four herbal formulas have been used to treat *qi* deficiency syndromes or internal heat syndrome, and the clinical indications are quite different from those of BJJG.

The result that rokumigan containing six crude drugs failed to suppress ear swelling in NC mice suggests that the additional two crude drugs, which are not contained in rokumigan but in BJJG, play an important role in the suppression of AD-like symptoms. The extract prepared from the mixture of ophiopogon tuber and schisandra fruit exhibited a suppressive effect on AD-like symptoms in NC mice, whereas rokumigan+ophiopogon tuber and rokumigan+schisandra fruit were not effective against dermatitis. It is suggested that the combination of ophiopogon tuber and schisandra fruit may

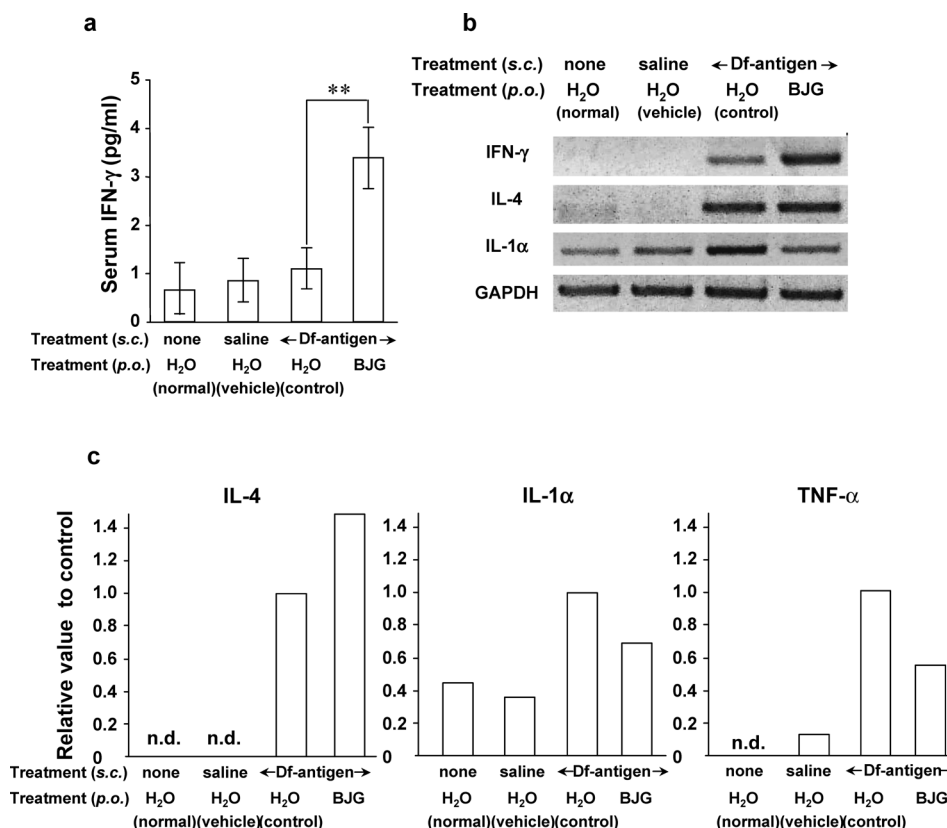


Fig. 3. Effect of Bakumijiogan (BJG) on Serum Concentration and Ear mRNA Levels of Cytokines in NC/Jic Mice Topically Injected with Df-Antigen

(a) Serum concentration of interferon (IFN)- γ on day 17 of NC/Jic mice are shown. See the legend for Fig. 2 for murine groups. Detection limit was >0.5 pg/ml. The values of 2 mice in each normal, vehicle and control group were undetectable, and data were regarded as zero. Data are represented as mean $\pm$ S.E. ($n=7$). ** $p<0.01$ vs. control group. (b) mRNA levels in the ear of NC/Jic mice evaluated by RT-PCR. (c) mRNA levels in the ear of NC/Jic mice evaluated by real-time RT-PCR. Data were calibrated by GAPDH mRNA expression, and shown as relative values to the control group.

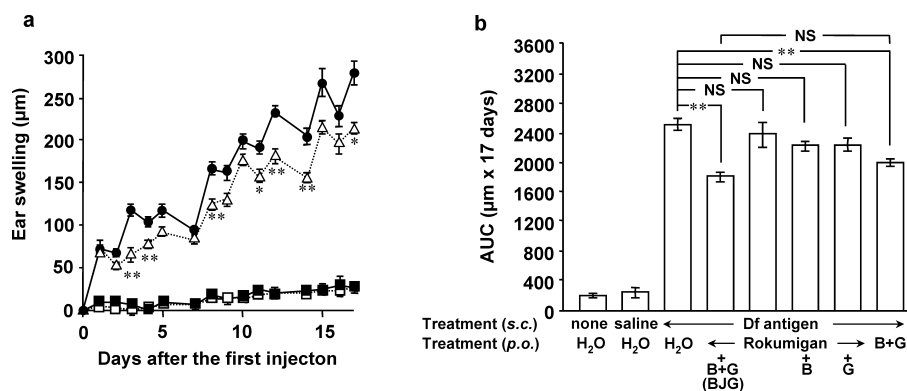


Fig. 4. Effect of the Herbal Constituents of Bakumijiogan (BJG) on Ear Swelling in NC/Jic Mice Topically Injected with Df-Antigen

(a) Consecutive ear swelling of NC/Jic mice treated with ophiopogon tuber (B) and schisandra fruit (G) from days -7 to 17 . Open square, normal group (no s.c. treatment and oral treatment with H₂O); closed square, vehicle group (s.c. injection of saline and oral treatment with H₂O); closed circle, control group (s.c. injection of Df-antigen and oral treatment with H₂O); open triangle, B+G group (s.c. injection of Df-antigen and oral treatment with B+G, 0.456 g/kg/d). (b) AUC of ear swelling of NC/Jic mice treated with rokumigan, rokumigan+B+G (equal to BJG), rokumigan+B, rokumigan+G, and B+G. The AUC was calculated by the trapezoid rule. Data are represented as mean $\pm$ S.E. ($n=7$). ** $p<0.01$ vs. control group. NS, not significant.

be important to suppress AD-like symptoms. In the TCM theory, ophiopogon tuber and schisandra fruit are used to promote body fluid production,⁶⁾ and they are sometimes prescribed together in several TCM formulas (e.g., shomyakusan and seisyoeckito). The present study could provide evidence for the combination and synergism of these two crude drugs. Previous studies have shown that each single crude drug would have anti-inflammatory effects in mice: water extract of ophiopogon tuber was reported to exhibit an inhibitory ef-

fect on xylene-induced ear swelling and carrageenan-induced paw edema.²³⁾ Schizandrin, a constituent of schisandra fruit, was shown to suppress passive anaphylaxis and itching.²⁴⁾ Little is known about the synergistic effects of these two crude drugs, and further studies are required.

In conclusion, BJG suppressed AD-like symptoms in NC mice repeatedly injected with Df-antigen into the ear auricle by correcting the Th1/Th2 imbalance skewed toward Th2. The combination of two herbal ingredients, ophiopogon

tuber and schisandra fruit, mainly contributed to the effects of BJG.

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