Therapeutic Effects of Gyokuheifusan on NC/Nga Mouse Model of Allergic Dermatitis

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Gyokuheifusan (GHS) is a traditional Chinese medicine (TCM) formula used to treat various allergic diseases; however, there is little information about its clinical efficacy or mechanism of action. We previously showed that GHS down-regulates over-production of IgE and interleukin (IL)-4 in a mouse model of ovalbumin (OVA)-induced asthma, a major Th2-dominant disease. Here, we investigated the effect of GHS in an NC/Nga mouse model of atopic dermatitis (AD) induced by mite antigen. NC/Nga mice were immunized with an intradermal injection of mite antigen extract twice a week from 6 to 12 weeks of age. GHS (3.0 g/kg) was orally administered daily to the GHS-treated group for 6 weeks, and dermatitis score and total serum IgE level were measured sequentially. Spleen was harvested at 13 weeks of age, and the levels of cytokines released from splenocytes (interferon (IFN)-γ, IL-4 and IL-5) and IgG1/IgG2a levels in sera were measured by ELISA. Mice treated with GHS showed milder dermatitis than the disease-control group and the increase of total serum IgE level tended to be suppressed by GHS. In addition, GHS treatment suppressed the production of IL-4 and IgG1, though it did not affect the production of IFN-γ and IgG2a; *i.e.*, GHS normalized the Th1/Th2 balance. These results suggest that GHS inhibits the development and reduces the severity of AD, at least in this model.

Key words —— Gyokuheifusan, atopic dermatitis, Th1/Th2 balance

INTRODUCTION

Atopic dermatitis (AD) is a common inflammatory skin disease affecting 10–20% of children and 1–3% of adults worldwide.[1] It has a complex and chronic pathogenesis that provides many potential cellular and molecular targets for therapeutic intervention, but may also include redundant pathways.[2] In addition, although corticosteroids and calcineurin inhibitors are used in the treatment of AD,[3,4] they remain only palliative.

Recently, traditional Chinese medicines (TCM) have attracted considerable attention. Since they usually contain many different herbal medicines, interactions among their constituents are likely to play an important role in their effects. Because of this complexity, it can be difficult to identify the active components and to elucidate the mechanisms of their actions. Relatively little scientific evidence is available on the efficacy of TCM.

Gyokuheifusan (GHS; Yu-Ping-Feng-San in Chinese, Jade Windscreen Powder in English) is a TCM formula that can be used to treat allergic diseases. Three herbal medicines are contained in GHS: root of *Astragalus membranaceus* Bunge, rhizome of *Atractylodes ovata* Decandille and root of *Saposhnikovia divaricata* Schischkin. In TCM theory, GHS strengthens protective *qi* from the lungs to guard the exterior of the body, increasing patients' protection against invasion by external pathogenic influences.[5] GHS is widely prescribed to treat respiratory tract diseases, such as respiratory infection, allergic rhinitis, bronchial asthma, and other such conditions in China, Japan, and Korea. In addition, GHS is believed to increase vigor.[5] In pharmacological studies of GHS, an immunomodulatory effect, and improvements in an allergic asthma model in mice and an allergic rhinitis model in guinea pigs were found.[6–8] The daily adult human dosage of GHS is 1.5 g/day, in this study, we treated with 100-times amount of human dosage (3 g/kg) experimen-

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tally.

NC/Nga mouse is a well-established animal model of human AD. NC/Nga mice raised in air-uncontrolled conventional circumstances spontaneously suffer from AD-like skin lesions with a marked elevation in plasma levels of total IgE, whereas NC/Nga mice maintained under specific pathogen-free (SPF) conditions do not show any clinical symptoms.[9,10] Under SPF conditions, AD-like skin lesions can be also caused by continuous administration of hapten. NC/Nga mice are used in several AD models, including spontaneous AD model, mite antigen-induced AD model[11–13] and picryl chloride (PiCl)-induced AD model.[14–16]

We previously examined the immunomodulatory effects of GHS in a mouse model of ovalbumin (OVA)-induced asthma, a major Th2-dominant-disease.[5] We found that GHS inhibits the development and reduces the severity of asthma through normalization of the interferon (IFN)-γ/interleukin (IL)-4 (Th1/Th2) balance. Therefore, in this study, we focused on the immunomodulatory effects of GHS in a mouse model of mite antigen-induced AD model, to determine whether GHS might be effective against other Th2-dominant diseases.

MATERIALS AND METHODS

Animals —— Six-week-old male SPF NC/Nga mice, weighing 16–18 g were purchased from Sankyo Laboservice Co. (Tokyo, Japan). They were housed in a SPF room kept at a temperature of $23 \pm 1^{\circ}C$ and a relative humidity of $55 \pm 5\%$ under a 12 hr light-dark cycle. They had free access to tap water and sterilized experimental normal diet. The mice were separated into 3 groups, *i.e.*, normal, disease-control, and GHS-treated groups ($n = 5$ in each group). Animals were treated and/or handled according to the Recommendations for Handling of Laboratory Animals for Biomedical Research, compiled by the Committee on the Safety and Ethical Handling Regulations for Laboratory Animals Experiments, Tokyo University of Science. All experimental procedures mentioned below were conducted in accordance with institutional guidelines for the care and use of laboratory animals in research.

Reagents —— GHS used in this experiment was a dried brown-yellow powder extract with a slightly bitter-sweet taste, and was kindly provided by Iskra Industry Co., Ltd. (Tokyo, Japan). The daily dose of GHS (1.5 g) consisted of the extract of a mixture of the following herbs: Astragalus Root (6 g), Atractylodes Rhizome (2 g), and Saposhnikovia Root (2 g), all of which are registered in Japanese Pharmacopoeia XV.[17] These medicinal herbs were boiled in 6 times their weight of H_2O for 60 min, then the mixture was filtered and the decoction was lyophilized. Quantitative analysis of marker compounds of each medicinal herb in GHS powder (calycosin from Astragalus Root, atratylenolide III from Atractylodes Rhizome, and 4′-*O*-β-D-glucosyl-5-*O*-methylvisamminol from Saposhnikovia Root) by 3D-HPLC chromatogram has been reported by Makino *et al.*[8]

Dermatophagoides fainae crude extract (mite antigen, diagnostic agent, Torii Pharmaceutical Co., Ltd., Tokyo, Japan) was used as a mite antigen.

The following antibodies and regents were used in this study. For measurement of immuno globulins, anti-mouse IgE monoclonal antibody (mAb), horseradish peroxidase (HRP)-conjugated anti-mouse IgE mAb, mouse IgE calibrator, HRP-conjugated anti-mouse IgG2a mAb, HRP-conjugated anti-mouse IgG1 mAb, and mouse reference serum were purchased from Bethyl Laboratories, Inc. (Montgomery, TX, U.S.A.). For measurement of cytokines, anti-mouse IFN-γ mAb (XMG1.2), biotin-conjugated anti-mouse IFN-γ mAb (R4-6A2), anti-mouse IL-4 mAb (11B11), biotin-conjugated anti-mouse IL-4 mAb (BVD6-24G2), anti-mouse IL-5 mAb (TRFK 5), biotin-conjugated anti-mouse IL-5 mAb (TRFK 4), and recombinant mouse cytokines (IFN-γ, IL-4 and IL-5) were purchased from eBioscience (San Diego, CA, U.S.A.). For immunofluorescence studies, the following mAbs were used: phycoerythrin (PE)-conjugated anti-mouse CD3 mAb (17A2), which recognizes T cell receptor-associated complex present on all mature T cells; phycoerythrin-cyanine 5 (PE-Cy5)-conjugated anti-mouse CD4 mAb (H129.19), which recognizes protein on helper T cells; fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD8a mAb (53-6.7), which recognizes protein on cytotoxic T cells; FITC-conjugated anti-mouse CD19 mAb (1D3), which recognizes protein on B cells; FITC-conjugated anti-mouse CD49b mAb (DX5), which recognizes protein on NK cells. All reagents used in this study were of reagent grade.

Induction of AD —— Induction of AD in NC/Nga mice was performed as described by Sasakawa *et al.*[11] Briefly, we injected 10 µl of the mite anti-

gen extract intradermally at the ventral side of the right ear of disease-control and GHS-treated mice twice a week from 6 to 12 weeks of age (total 12 times). The mice of the normal group received mock sensitization with vehicle (50% glycerin in 5% NaCl aq.). The clinical symptoms were evaluated twice a week by using a clinical severity grading score for dermatitis of the right ear: 0 (none), 1 (mild; partial hyperplasia, edema and hemorrhage), 2 (moderate; hyperplasia, edema and hemorrhage in whole ear lobe), 3 (severe; loss of the ear).

Treatment with GHS —— GHS used for treatment was suspended in pure water and given in a volume of 25 ml/kg orally to the GHS-treated group (3.0 g/kg, 100-times amout of human daily dosage) every day from 3 days before the first sensitization at 6 weeks of age to the day before sacrificing at 12 weeks of age. The normal group received the same volume of pure water.

Collection of Blood —— According to the procedure described by Matsuda *et al.*,[9] blood was collected from retro-orbital plexus with glass capillary tubes at 24 hr after mite sensitization, and the serum was separated. To determine the time-course of changes in total IgE, blood samples were collected every week. The total IgE was measured by ELISA.

Splenocyte Preparation and Cytokine Measurement —— Levels of IFN-γ, IL-4 and IL-5 produced by splenocytes were measured as described by Fang *et al.*[5] Briefly, the mice were anaesthetized with diethyl ether and the spleen was excised at 13 weeks of age. The spleen cells (7×10^6 cells/ml) from normal or immunized mice were suspended in 10% FBS/RPMI1640 (GIBCO Invitrogen Co., Grad Island, NY, U.S.A.) culture medium in 96-well flat-bottomed plates (Corning Inc., Corning, NY, U.S.A.) and then cultured in the presence of mite antigen (1.5 μg/ml) at 37°C in a CO_2 incubator. The supernatant was collected after 96 hr for measurement of cytokine production by splenocytes, which is according to the Mutou's procedure,[18] and stored at −80°C for quantitative analysis. The levels of cytokines (IFN-γ, IL-4 and IL-5) in the culture supernatant were measured by ELISA.

ELISA Procedures —— Production of total IgE, IgG1, IgG2a and cytokines (IFN-γ, IL-4 and IL-5) was measured by ELISA using appropriate antibodies as described by Fang *et al.*[19] Briefly, flat-bottomed 96-well Maxisorp plates were coated with purified capture IgE mAb, IFN-γ mAb, IL-4 mAb or IL-5 mAb overnight at 4°C. The primary mAbs were discarded and the plates were blocked with blocking buffer [1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS)] for 1 hr at room temperature, then washed 3 times with wash buffer (0.05% Tween 20 in PBS). Appropriately diluted samples or IgE, IFN-γ, IL-4 and IL-5 standards were added and the plates were incubated for 2 hr at room temperature. The supernatants were discarded and the wells were washed 3 times with wash buffer. HRP-conjugated anti-mouse IgE mAb or biotin-labeled IFN-γ, IL-4 or IL-5 mAbs were added and the plates were further incubated for 1 hr at room temperature. The supernatants were discarded and the wells were washed 5 times with wash buffer, then avidin-HRP (BD Biosciences Pharmingen, San Diego, CA, U.S.A.) was added, and the plates were incubated for 30 min at room temperature. In the case of measuring IgE, this step was omitted, as the secondary IgE antibody was already labeled with HRP. Next, the wells were washed, and tetramethylbenzidine substrate solution (Pharmingen, San Diego, CA, U.S.A.) was added. The color was allowed to develop for 10–30 min in the dark before the reaction was quenched with a stop solution (5 N H_2SO_4). For measurement of IgG1 and IgG2a, direct ELISA was used, *i.e.*, flat-bottomed 96-well Maxisorp plates were coated with standard serum and sample sera, and detected by HRP-conjugated anti-mouse IgG1 mAb or HRP-conjugated anti-mouse IgG2a mAb. The optical density (OD) values were read at 450 nm and the sample concentrations of Igs and cytokines were determined according to the standard curve.

Histological Analysis —— Ear lobes were excised from each group at 13 weeks of age, fixed with 10% phosphate-buffered formalin, and embedded in paraffin. Thin sections were cut and stained with hematoxylin and eosin.

Phenotyping of Lymphocytes —— Immunophenotyping of splenic lymphocytes was carried out using a FACS-LSR flow cytometer (Becton Dickinson, San Jose, CA, U.S.A.). Splenocytes were harvested from each mouse at 13 weeks of age. The cells were suspended in RPMI1640-based buffer (pH 7.4) at a concentration of 2×10^6 cells/ml for use as samples. Cells were stained with fluorochrome-conjugated antibodies for 30 min. After the cells were washed, analysis of 10000 lymphocyte events per tube was performed using Cell Quest software (Becton Dickinson).

Statistical Analysis —— Values for all measurements were expressed as means ±S.E. For dermatitis scores and IgE levels, the significance of differ-

ences between the disease-control group and GHS-treated group were examined using one-way analysis of variance (ANOVA) followed by pairwise comparisons with Bonferroni's *post hoc* analysis. Comparisons of cytokine production were made using the unpaired Student's *t*-test. A probability value of less than 0.05 was considered to be statistically significant.

RESULTS

Attenuation of Dermatitis by GHS

Dermatitis was induced in NC/Nga mice by injection of mite antigen. One week after the first immunization, erythema and scars began to appear in the mite antigen-treated mice, followed by hyperplasia, edema and hemorrhage in their ears, and finally loss of the ears. These clinical signs and symptoms were assessed in terms of a clinical severity score, as shown in Fig. 1A. The clinical severity score for dermatitis of the right ear of the disease-control group was increased by repeated treatment with mite antigen. The severity score was significantly lower in GHS-treated mice.

Time-dependent changes in serum total IgE levels are shown in Fig. 1B. The level of serum total IgE was increased from 2 weeks after the first immunization in the disease-control group compared with the normal group. Consistent with the change in dermatitis score, the increase of serum total IgE tended to be suppressed by treatment with GHS.

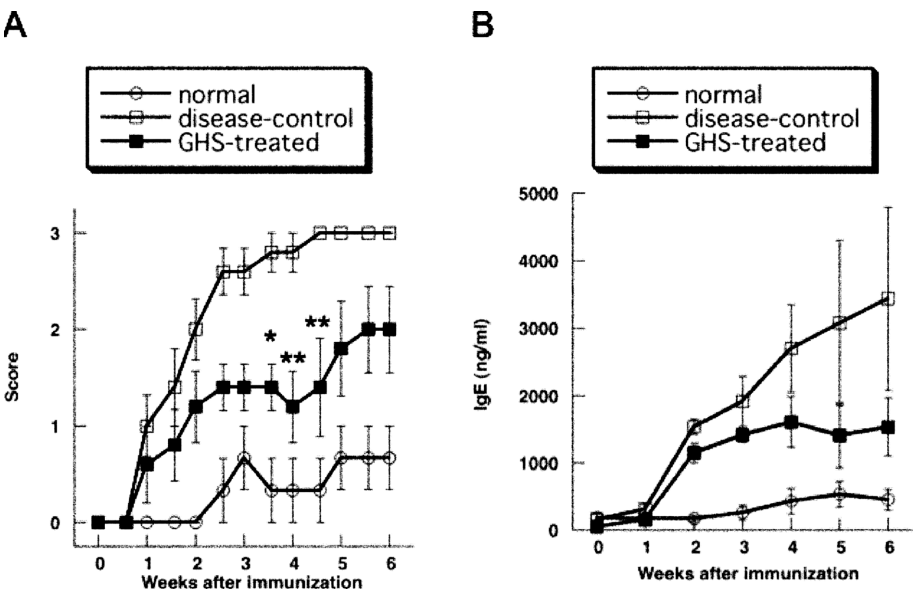

Fig. 1. Effect of GHS on Dermatitis Score and Total Serum IgE Level in NC/Nga Mice Treated with Mite Antigen

[A] Clinical severity in NC/Nga mice was scored twice a week in individual mice, as follows: 0 (none), 1 (mild; partial hyperplasia, edema and hemorrhage), 2 (moderate; hyperplasia, edema and hemorrhage in whole ear lobe), 3 (severe; loss of the ear). [B] Total serum IgE was obtained once a week from each mouse and IgE was assayed by means of ELISA. Each value represents the mean ±S.E. of 5 animals. Significant differences from the disease-control group are indicated with $^{*}p < 0.05$, $^{**}p < 0.01$.

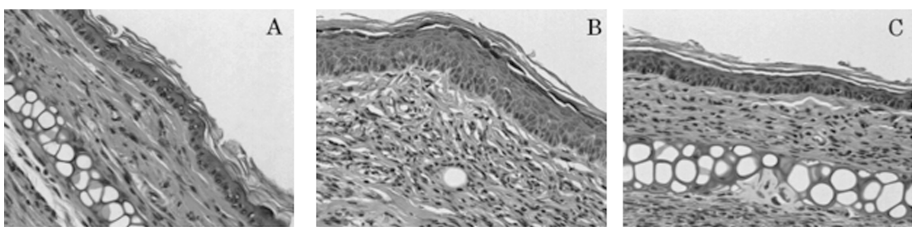

Fig. 2. Histopathological Images of NC/Nga Mice Treated Repeatedly with Mite Antigen

Ear lobes were excised 7 weeks after the first sensitization. Tissue sections were stained with hematoxylin and eosin. [A] normal; [B] disease control; [C] GHS-treated.

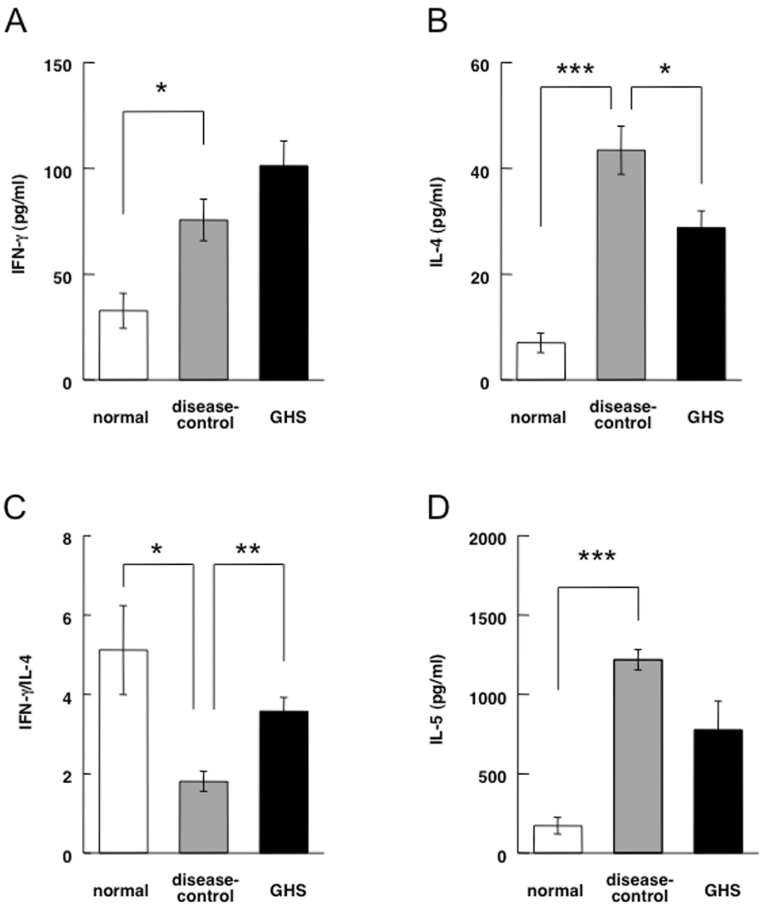

Fig. 3. Effects of GHS on the Levels of IL-4, IL-5, and IFN-γ, and on the IFN-γ/IL-4 (Th1/Th2) Ratio in NC/Nga Mice

Spleen was harvested 7 weeks after the first sensitization. Splenocytes (1.4×10^6 cell/well) were cultured in the presence of mite antigen (5 µl/well) at 37°C in a CO_2 incubator. The supernatants were collected at 96 hr for measurements of IL-4, IL-5, and IFN-γ by means of ELISA. [A] IFN-γ, [B] IL-4, [C] IFN-γ/IL-4 ratio, [D] IL-5. Each value represents the mean ±S.E. of 5 samples. Significant differences are indicated with $^{*}p < 0.05$, $^{***}p < 0.001$.

Histological Analysis

Histological images of ear lobes are shown in Fig. 2. Repeated treatment with mite antigen caused potent inflammatory changes, including thickening of the epidermis, and fibrosis in the dermis. Accumulation of inflammatory cells, such as lymphocytes, eosinophils and neutrophils, was observed in the ear lobes, compared with the vehicle-treated ear lobes. In contrast, administration of GHS clearly attenuated these signs of inflammation.

Effect of GHS on Production of Cytokines

AD is characterized by Th2-dominant response, and IgE production is mainly regulated by IL-4 and IFN-γ released from helper T cell (Th cell). To investigate changes in Th1/Th2 balance, the levels of cytokines released from splenocytes (IFN-γ, IL-4 and IL-5) were measured. Production of IFN-γ (Th1-type cytokine) was higher in the disease-control group than the normal group, and there was no significant difference between the disease-control group and the GHS-treated group (Fig. 3A). On the other hand, production of IL-4 (Th2-type cytokine) was increased in the disease-control group compared with the normal group, and GHS treatment significantly inhibited the increase of IL-4 (Fig. 3B). These results were reflected in the IFN-γ/IL-4 (Th1/Th2) balance. As shown in Fig. 3C, the Th1/Th2 balance was shifted toward Th2-dominant in the disease-control group, whereas the balance was normalized in the GHS-treated group. Furthermore, the production of IL-5 (Th2-type cytokine) was increased significantly in the disease-control group compared with the normal group, though GHS treatment caused no significant change in this case (Fig. 3D).

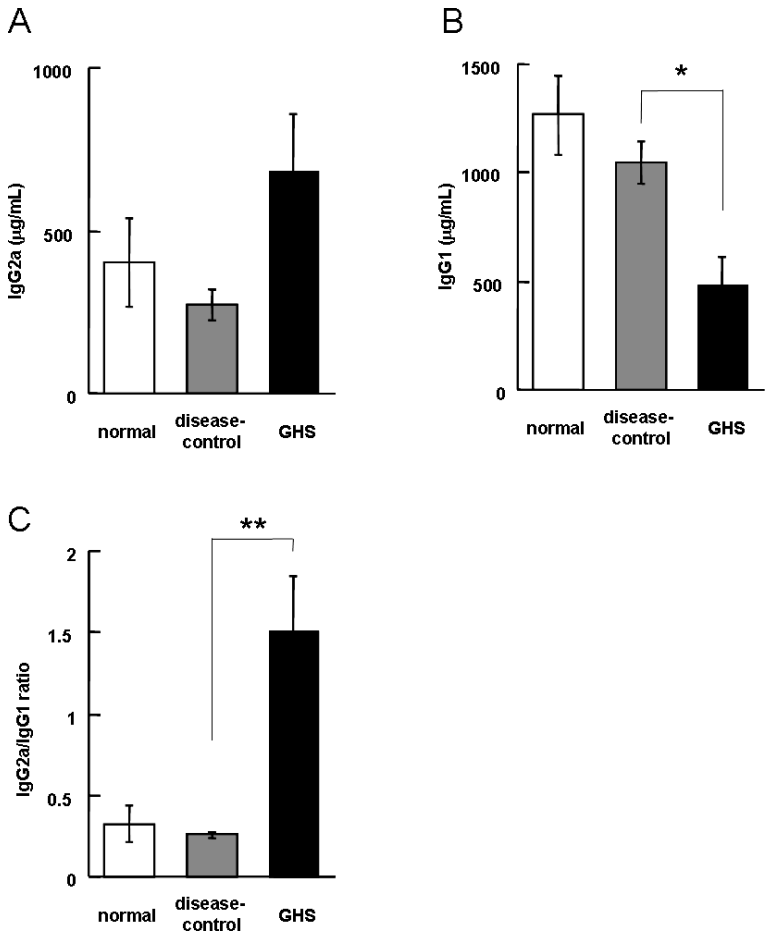

Fig. 4. Effects of GHS on the Levels of IgG2a and IgG1 Titer in Serum, and on the IgG2a/IgG1 (Th1/Th2) Ratio in NC/Nga Mice

Sera were collected 7 weeks after the first sensitization. The levels of IgG1 and IgG2a in serum were measured by ELISA. [A] IgG2a, [B] IgG1, [C] IgG2a/IgG1 ratio. Each value represents the mean ±S.E. of 5 samples. Significant differences are indicated with $^{*}p < 0.05$, $^{**}p < 0.01$.

Effect of GHS on Production of IgG Subset

For further investigation of Th1/Th2 balance, the levels of IgG1 and IgG2a in sera were measured. Production of IgG2a (Th1-type) was not changed by treatment with mite antigen or GHS (Fig. 4A). On the other hand, IgG1 (Th2-type) production was not changed by mite antigen sensitization in disease-control group, whereas a significant suppression of IgG1 production was found in GHS-treated group (Fig. 4B). As shown in Fig. 4C, the IgG2a/IgG1 (Th1/Th2) balance was not changed by mite antigen sensitization in disease-control group, however, it was shifted toward Th1-dominant in GHS-treated group.

Effect of GHS on Population of Splenic Lymphocytes

As shown in Fig. 5, no significant differences in any lymphocyte populations of pan-T cell, helper T cell, cytotoxic T cell, pan-B cell, and pan-NK cell were observed by treatment with mite antigen or GHS.

DISCUSSION

We have previously reported that GHS ameliorates the pathologies of an OVA-induced asthma model in mice. Asthma is a common chronic lung disease in industrialized countries, and is characterized by the production of large quantities of IgE antibody by B cells and a decrease of the IFN-γ/IL-4 (Th1/Th2) ratio. To examine further the effect of GHS on Th2-dominant disease, we used NC/Nga mouse, which is a widely used AD model, in this work. This is the first report to provide evidence that GHS has an ameliorative effect on the development of the pathology in NC/Nga mice.

NC/Nga mice develop AD-like skin lesions and clinical features similar to those of patients with AD

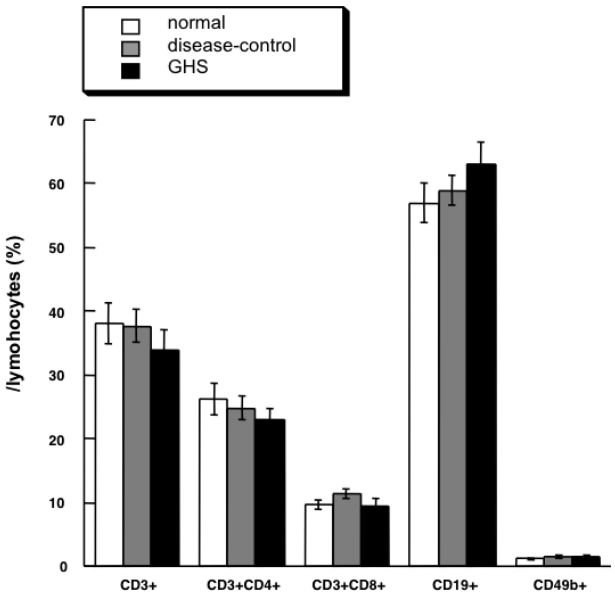

Fig. 5. Effects of GHS on Phenotype of Splenic Lymphocytes in NC/Nga Mice

Spleen was harvested 7 weeks after the first sensitization. Splenocytes (2×10^6 cells/ml) were suspended in RPMI-based buffer and stained with fluorochrome-conjugated antibodies. Phenotypes of lymphocytes were analyzed by flow cytometry. Each value represents the mean ±S.E. of 5 samples.

in response to repeated injections of mite antigens under SPF conditions.[11] Thus, we examined the effects of GHS on this animal model. We found that GHS ameliorates the AD-like skin lesions in this model. Sensitization by mite antigen caused erythema and scars followed by hyperplasia, edema and hemorrhage in the antigen-treated ears, and finally loss of the ears. Treatment with GHS suppressed the development of erythema and scars. Furthermore, the histological pathology, especially the infiltration of granulocytes, was improved by the treatment with GHS.

In addition, GHS normalized the Th1/Th2 ratio and tended to decrease the level of IgE. AD is a Th2-dominant disease, at least in the initial phase, in which lymphocytes infiltrating the skin mainly produce IL-4, IL-5 and IL-10.[20] The NC/Nga mouse model also develops a Th2-dominant response, with overproduction of Th2-type cytokines (IL-4, IL-5 and IL-10), Th2-specific chemokines,[9,21,22] and IgE antibody. We found that the overproduction of IgE antibody and the levels of IL-4, IL-5 and IFN-γ were increased by mite sensitization, and GHS treatment suppressed the production of IL-4, IgG1 and, to some extent, IgE antibody, though it did not affect the production of IFN-γ, IL5, or IgG2a. Thus, GHS could normalize the Th1/Th2 balance and regulate the production of cytokines and IgGs. Since AD is frequently associated with elevated levels of serum IgE against many kinds of encroaching allergens, it has been speculated that IgE antibody may be involved in the development of AD.[23] IgE synthesis in B cells is also regulated by IL-4 and IFN-γ.[24,25] These cytokines are mainly released from helper T cells. However, the percentages of helper T cells ($CD3^+CD4^+$), cytotoxic T cells ($CD3^+CD8^+$), B cells ($CD19^+$) and NK cells (pan-NK^+) were not changed by treatment with mite antigen or GHS, indicating that GHS did not affect the population of splenic lymphocytes. Thus, these observations suggest that GHS acts on cytokine production, but not on the lymphocyte population. The effect of GHS on Th1/Th2 balance may be more important in this model. IL-5 is a Th2-cytokine which is known to be involved in differentiation of B cells into plasma cells, and proliferation/activation and migration of eosinophils. Therefore, IL-5 plays an important pathogenic role in AD.[26,27] Consistent with these observations, the level of IL-5 was increased in the mite antigen-treated group. However, GHS treatment did not inhibit this response. Thus, GHS treatment did not markedly suppress the increase of IgE.

These results are consistent with those of other studies by Yang *et al.*[28] and Zhao *et al.*[29] Astragalus Root (one of the main materials of GHS) reduced the secretion of IL-4 by regulating the shift of Th2 to Th1, and the total IgE produced by B cells decreased accordingly. In additional, Astragalus Root significantly promoted IFN-γ, IgG1, IgG2, and IgG3 production from peripheral blood monocytes *in vitro*, but had no significant effect on the production of IL-4 or IgG4, suggesting that it can modulate Th1/Th2 balance and enhance the immune function of AD sufferers by improving Th1 deficiency.

In conclusion, our results indicate that GHS has ameliorating effects in this mite antigen-induced AD model. We showed that GHS tended to suppress the overproduction of IgE, with normalization of the Th1/Th2 balance. These results indicate that GHS inhibited the development and reduced the severity of atopic dermatitis. GHS is commonly used to treat to respiratory diseases in clinical practice in various oriental countries, and there is currently much interest in the mechanisms of its effects on the immune system. An understanding of these mechanisms may provide a pharmacological basis for more effective clinical application of GHS and possibility for its application to the treatment to other diseases.

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REFERENCES

1) Leung, D. Y., Boguniewicz, M., Howell, M. D., Nomura, I. and Hamid, Q. A. (2004) New insights into atopic dermatitis. *J. Clin. Invest.*, **113**, 651–657.
2) Inoue, J. and Aramaki, Y. (2007) Suppression of skin lesions by transdermal application of CpG-oligodeoxynucleotides in NC/Nga mice, a model of human atopic dermatitis. *J. Immunol.*, **178**, 584–591.
3) Furue, M., Terao, H., Moroi, Y., Koga, T., Kubota, Y., Nakayama, J., Furukawa, F., Tanaka, Y., Katayama, I., Kinukawa, N., Nose, Y. and Urabe, K. (2004) Dosage and adverse effects of topical tacrolimus and steroids in daily management of atopic dermatitis. *J. Dermatol.*, **31**, 277–283.
4) Yamamoto, M., Haruna, T., Yasui, K., Takahashi, H., Iduhara, M., Takaki, S., Deguchi, M. and Arimura, A. (2007) A novel atopic dermatitis model induced by topical application with dermatophagoides farinae extract in NC/Nga mice. *Allergology International*, **56**, 139–148.
5) Fang, S. P., Tanaka, T., Tago, F., Okamoto, T. and Kojima, S. (2005) Immunomodulatory effects of gyokuheifusan on INF-gamma/IL-4 (Th1/Th2) balance in ovalbumin (OVA)-induced asthma model mice. *Biol. Pharm. Bull.*, **28**, 829–833.
6) Makino, T. (2005) [Pharmacological properties of Gyokuheifusan, a traditional Kampo medicinal formula] *Yakugaku Zasshi*, **125**, 349–354 (in Japanese).
7) Makino, T., Sasaki, S. Y., Ito, Y. and Kano, Y. (2005) Pharmacological properties of traditional medicine (XXX): effects of Gyokuheifusan on murine antigen-specific antibody production. *Biol. Pharm. Bull.*, **28**, 110–113.
8) Makino, T., Ito, Y., Sasaki, S. Y., Fujimura, Y. and Kano, Y. (2004) Preventive and curative effects of Gyokuheifu-san, a formula of traditional Chinese medicine, on allergic rhinitis induced with Japanese cedar pollens in guinea pig. *Biol. Pharm. Bull.*, **27**, 554–558.
9) Matsuda, H., Watanabe, N., Geba, G. P., Sperl, J., Tsudzuki, M., Hiroi, J., Matsumoto, M., Ushio, H., Saito, S., Askenase, P. W. and Ra, C. (1997) Development of atopic dermatitis-like skin lesion with IgE hyperproduction in NC/Nga mice. *Int. Immunol.*, **9**, 461–466.
10) Suto, H., Matsuda, H., Mitsuishi, K., Hira, K., Uchida, T., Unno, T., Ogawa, H. and Ra, C. (1999) NC/Nga mice: a mouse model for atopic dermatitis. *Int. Arch. Allergy Immunol.*, **120**, 70–75.
11) Sasakawa, T., Higashi, Y., Sakuma, S., Hirayama, Y., Sasakawa, Y., Ohkubo, Y., Goto, T., Matsumoto, M. and Matsuda, H. (2001) Atopic dermatitis-like skin lesions induced by topical application of mite antigens in NC/Nga mice. *Int. Arch. Allergy Immunol.*, **126**, 239–247.
12) Gao, X. K., Nakamura, N., Fuseda, K., Tanaka, H., Inagaki, N. and Nagai, H. (2004) Establishment of allergic dermatitis in NC/Nga mice as a model for severe atopic dermatitis. *Biol. Pharm. Bull.*, **27**, 1376–1381.
13) Gao, X. K., Fuseda, K., Shibata, T., Tanaka, H., Inagaki, N. and Nagai, H. (2005) Kampo Medicines for Mite Antigen-Induced Allergic Dermatitis in NC/Nga Mice. *Evid. Based Complement. Alternat. Med.*, **2**, 191–199.
14) Hirasawa, Y., Ori, K., Ohtsu, S., Matsui, Y., Shimizu, M., Kyuki, K. and Higo, S. (2003) Analysis of scratching behavior in a picryl chloride induced atopic dermatitis model in mice. *Arerugi*, **52**, 1014–1021.
15) Kato, Y., Mizuguchi, K. and Mochizuki, H. (2005) A novel benzoimidazole derivative, M50367, modulates helper T type I/II responses in atopic dermatitis mice and intradermal melanoma-bearing mice. *Biol. Pharm. Bull.*, **28**, 78–82.
16) Sakai, T., Kogiso, M., Mitsuya, K. and Yamamoto, S. (2004) Effect of genistein on immune responses in NC/Nga mice used as an animal model for atopic dermatitis. *Protein Research Japan*, **7**, 130–136.
17) Japanese Pharmacopoeia Convention (2006) Japanese Pharmacopoeia, XV Edition, Ishiyaku Shuppan, Tokyo.
18) Mutou, Y., Ibuki, Y. and Kojima, S. (2007) Immunomodulatory effects of ultraviolet B irradiation on atopic dermatitis in NC/Nga mice. *Photodermatol. Photoimmunol. Photomed.*, **23**, 135–144.
19) Fang, S. P., Muto, Y., Tago, F., Simura, N. and Kojima, S. (2006) Effect of repeated small-dose gamma-ray irradiation on atopic dermatitis in NC/Nga mice. *J. Health Sci.*, **52**, 406–411.
20) Grewe, M., Bruijnzeel-Koomen, C. A., Schöpf, E., Thepen, T., Langeveld-Wildschut, A. G., Ruzicka, T. and Krutmann, J. (1998) A role for Th1 and Th2 cells in the immunopathogenesis of atopic dermatitis. *Immunol. Today*, **19**, 359–361.
21) Yagi, R., Nagai, H., Iigo, Y., Akimoto, T., Arai, T. and Kubo, M. (2002) Development of atopic dermatitis-like skin lesions in STAT6-deficient NC/Nga mice. *J. Immunol.*, **168**, 2020–2027.
22) Vestergaard, C., Yoneyama, H., Murai, M.,

Nakamura, K., Tamaki, K., Terashima, Y., Imai, T., Yoshie, O., Irimura, T., Mizutani, H. and Matsushima, K. (1999) Overproduction of Th2-specific chemokines in NC/Nga mice exhibiting atopic dermatitis-like lesions. *J. Clin. Invest.*, **104**, 1097–1105.

23) Matsumoto, M., Ra, C., Kawamoto, K., Sato, H., Itakura, A., Sawada, J., Ushio, H., Suto, H., Mitsuishi, K., Hikasa, Y. and Matsuda, H. (1999) IgE hyperproduction through enhanced tyrosine phosphorylation of Janus kinase 3 in NC/Nga mice, a model for human atopic dermatitis. *J. Immunol.*, **162**, 1056–1063.

24) Cherwinski, H. M., Schumacher, J. H., Brown, K. D. and Mosmann, T. R. (1987) Two types of mouse helper T cell clone. III. Further differences in lymphokine synthesis between Th1 and Th2 clones revealed by RNA hybridization, functionally monospecific bioassays, and monoclonal antibodies. *J. Exp. Med.*, **166**, 1229–1244.

25) Mosmann, T. R. and Coffman, R. L. (1989) TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu. Rev. Immunol.*, **7**, 145–173.

26) Kondo, S., Yazawa, H. and Jimbow, K. (2001) Reduction of serum interleukin-5 levels reflect clinical improvement in patients with atopic dermatitis. *J. Dermatol.*, **28**, 237–243.

27) Simon, D., Braathen, L. R. and Simon, H. U. (2004) Eosinophils and atopic dermatitis. *Allergy*, **59**, 561–570.

28) Yang, Q. X., Yang, J. Y., Qu, C. F., Li, D. J., Wang, H., Hao, X. Y. and Wang, L. R. (2002) *Chinese Journal of Microecology*, **14**, 17–18.

29) Zhao, X. D. (1997) *Chinese Journal of Practical Padiatrics*, **12**, 345–346.