Asthma, a common, chronic lung disease in industrialized countries, 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. Gyokuheifusan (GHS) is a classical formulation of traditional Chinese medicine (TCM) that is usually prescribed to prevent or treat respiratory tract diseases, such as respiratory infection and bronchial asthma. In order to evaluate the possible effectiveness of GHS on bronchial asthma, its immunomodulatory activity was examined in ovalbumin (OVA)-induced asthma model mice. All mice, except those in the normal group, were sensitized by intraperitoneal (IP) administration of OVA emulsified with Al(OH)3, and a second immunization was given 6 d later. After a further 13, 17 and 21d, mice were challenged with inhalation of aerosolized OVA solution, except for the normal group, which received mock sensitization using saline-Al(OH)3 emulsion and were challenged with an aerosol of saline without OVA. Allergen-specific IgE and total IgE in plasma were both significantly increased in the disease-control group. These increases were markedly blocked by GHS treatment. IFN-γ released by splenocytes was significantly increased after co-culture with OVA for 24 h, 48 h, and 72 h. GHS treatment further elevated the IFN-γ content compared with the disease-control group. The production of IL-4 was significantly increased when splenocytes were stimulated with OVA for 72 h, but this increase was blocked by GHS treatment, so that GHS returned the decreased IFN-γ/IL-4 (Th1/Th2) ratio of the disease-control group to the normal range. These results indicate that GHS may inhibit the development and severity of asthma.

Key words Gyokuheifusan (GHS); anti-allergic effect; asthma
effective.

Gyokuheifusan (GHS; Yu-Ping-Feng-San in Chinese) is a classical formula of traditional Chinese medicine (TCM), and 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 TCM, GHS is believed to increase human vigor and to enhance the protective function of the lungs, defending the body against invasion by external pathogenic influences. In this study, we focused on the immuno-regulatory effects of GHS on the Th1/Th2 balance in ovalbumin (OVA)-induced asthma model mice.

MATERIALS AND METHODS

Animal Experiments BALB/c mice (6 weeks old, female), weighing 15—20 g, were purchased from Sankyo Laboservice Co. (Tokyo, Japan). They were housed in plastic cages with sterilized wood-chip bedding and bred in animal rooms kept at a temperature of 23°C and a relative humidity of 55±10% under a 12-h light–dark cycle. They had free access to tap water and experimental normal diet (CE-2, CLEA Co., Ltd., Tokyo, Japan). The mice were separated into 3 groups, i.e., normal, disease-control and GHS-treated groups. 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.

Plant Material and 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). The extraction method was as follows. Astragalus Root 6 kg, Atractylodes Rhizome 2 kg, and Saposhnikovia Root 2 kg were mixed and boiled in 6 times their weight of water at 100 °C for 1 h, and then the liquid was centrifuged, filtered and lyophilized. The extract amounted to about 15% of the input herbs. Quantitative analysis of marker compounds of each medicinal herb in GHS powder (calycosin from Astragalus Root, atratylenolide III from Atractylodes Rhizome, and GMV from Saposhnikovia Root) by 3D-HPLC chromatogram has been reported by Makino et al., and the chemical structures of these compounds are shown in Fig. 1. We found that 0.8—1.5 mg of 4′-O-β-D-glucosyl-5-O-methylvisamminol (GMV, C_{22}H_{28}O_{10}) was present in each 1 g package of GHS powder. All drugs used for treatment were suspended in pure water and given in a volume of 25 ml/kg to mice by gastric gavage, while the same volume of pure water was given to the normal group.

OVA was used as an antigen. Al(OH)_3 (13 mg/ml) were mixed and i.v. administered to mice. Then, 13, 17 and 21 d later, mice were placed in a glass barrel for 30 min and challenged with inhalation of aerosolized OVA solution (10 mg/ml in saline) for excitation. The aerosol was generated by an Omron device (NE-U22, Omron Co.). Mice in the normal group received i.p. mock sensitization with saline-Al(OH)_3 emulsion and were challenged with an aerosol of saline without OVA.

Evaluation of the OVA-Specific IgE Antibody The serum OVA-specific IgE antibody level was evaluated by passive cutaneous anaphylaxis (PCA) reaction according to the method described by Bando et al. and Tai et al. with some modifications. Briefly, the disease-control and GHS-treated groups were sensitized i.p. with 100 µl of OVA solution (0.5 mg/ml in saline) emulsified in 150 µl of Al(OH)_3 at a total dosage of 250 µl. A second sensitization was given 6 d after the initial sensitization. Then, 13, 17 and 21 d later, mice were placed in a glass barrel for 30 min and challenged with inhalation of aerosolized OVA solution (10 mg/ml in saline) for excitation. The aerosol was generated by an Omron device (NE-U22, Omron Co.). Mice in the normal group received i.p. mock sensitization with saline-Al(OH)_3 emulsion and were challenged with an aerosol of saline without OVA.

Assay of Plasma Total IgE According to the procedure described by Watanabe et al., blood was collected from the retro-orbital plexus with glass capillary tubes at 1 h and 24 h post-final inhalation, and the plasma was stored at −80°C.
until quantitative analysis. To determine the time-course of changes in total IgE, blood samples were collected every 2 d from day 5 to day 38 after the start of the experiment. The total IgE was measured with an ELISA kit (Bethyl Laboratories, Inc.). The assay sensitivity was 3.9—250 ng/ml, and the samples were appropriately diluted.

**Splenocyte Preparation and Cytokine Assays** Levels of IL-4 and IFN-γ produced by splenocytes were measured as described by Wang et al. Briefly, the mice were anaesthetized and their spleens were excised 1 h or 24 h after the final inhalation. The spleen cells (5×10⁶ cells/ml) from normal or immunized mice were suspended in a 10% FBS/RPMI1640 culture medium and then cultured in the presence of OVA (1 ng/ml) at 37 °C in a CO₂ incubator. The supernatant was collected at 24 h, 48 h and 72 h for measurement of IL-4 and IFN-γ. The levels of cytokines (IL-4 and IFN-γ) in the culture supernatant were measured with ELISA kits (Pharmingen). For cytokine assays, flat-bottomed 96-well Maxisorp plates were coated with purified capture IL-4 mAb or IFN-γ mAb overnight at 4 °C. The primary mAbs were discarded and the plates were blocked with blocking buffer (1% BSA in PBS) for 1 h at room temperature. The plates were washed 3 times with wash buffer (0.05% Tween 20 in PBS) and blotted on a paper towel. Appropriately diluted samples or IL-4 and IFN-γ standards were added and incubated for 2 h at room temperature. The supernatants were discarded and the wells were washed 5 times with wash buffer. Biotin-labeled IL-4 or IFN-γ mAbs were added and incubated for 1 h at room temperature. The supernatant was discarded and the wells were washed 5 times with wash buffer, then Avidin-HRP was added, and the plates were incubated for 30 min at room temperature. The Avidin-HRP solution was collected and tetramethylbenzidine (TMB) substrate solution (Pharmingen, San Diego, CA, U.S.A.) was added after the wells had been washed 5 times with wash buffer. The color was allowed to develop for 10—30 min in the dark before the reaction was quenched with a stop solution (2 M H₂SO₄). The OD values were read at 450 nm and the sample concentrations were determined according to the standard curve. The assay sensitivities were 16—2000 pg/ml (IL-4 system) and 78—5000 pg/ml (IFN-γ system).

**Statistical Analysis** The data were subjected to statistical analysis and results were expressed as mean ± S.D. Significance of differences was determined by use of a two-tailed Student’s t-test. A probability value of less than 0.05 was considered to be statistically significant.

**RESULTS**

**Influence of the OVA-Specific IgE** In clinical practice, allergen-specific IgE (as demonstrated by skin testing or in vitro assays) is generally believed to be inextricably associated with the induction of allergic airway symptoms, and is used as a guide for environmental modification and immunotherapy. In this study, the size of the blue spot on the skin was assayed as a measure of OVA-specific IgE. Fig. 2 indicates the results of PCA analysis for OVA-specific IgE production in BALB/c mice. The disease-control group sensitized with OVA showed a significantly increased level compared with the non-sensitized normal group. The increase was significantly inhibited by GHS treatment.

**Fig. 2. Effects of Gyokuheifusan (GHS) on OVA-Specific IgE in OVA-Induced Asthma Model Mice**

The values represent means ± S.D. of 4 animals. Significant differences from the normal group are indicated with * (p < 0.01), and from the disease control group with + (p < 0.05).

**Fig. 3. Time-Dependent Changes in Total IgE Level in OVA-Induced Asthma Model Mice**

Total IgE in plasma post-final inhalation was assayed every two or three days from the 10th to 38th day. The values of the normal group were 255 ng/ml on day 8, 257 ng/ml on day 14, 205 ng/ml on day 18, and 278 ng/ml on day 24. The values represent means ± S.D. of 5 animals.

**Time-Course of Total IgE Induced by OVA** Time-dependent changes in the total IgE levels are illustrated in Fig. 3. The total IgE content in OVA-induced asthmatic mice was significantly increased from 20 d after the start of the experiment, reaching a maximum at around 30 d. Thereafter, the level gradually decreased up to 38 d. The values of the normal group were 255 ng/ml on day 8, 257 ng/ml on day 14, 205 ng/ml on day 18, and 278 ng/ml on day 24. The results indicate that BALB/c mice can develop an OVA-induced increase of plasma IgE.

**Effects of GHS on the Increase of Total IgE Induced by OVA** As shown in Fig. 4, the level of total IgE was significantly increased in the disease-control group compared with the normal group at 1 h post-final inhalation of OVA. The increase was significantly decreased by GHS treatment. At 24 h post-final inhalation, total IgE in the OVA-treated group remained high, though it was lower than that at 1 h post-final
all incubation times, indicating that GHS could stimulate level than in the OVA alone-treated (disease-control) group at 37 °C in a CO2 incubator. The supernatant was collected at 24 h, 48 h pended in a 10% FBS/RPMI1640 culture medium and then cultured in the presence of γ and 72 h for measurement of IFN-γ.

Effect of GHS on the increase of IFN-γ induced by OVA

Production of IFN-γ at 1 h-post-final inhalation was shown in Fig. 5. Production of IFN-γ was significantly increased at 1 h post-final inhalation when the splenocytes were stimulated with OVA for 24 h, 48 h or 72 h at 24 h post-final inhalation. The spleen cells (5 × 10⁶ cells/ml) from normal or immunized mice were stimulated with OVA for 72 h at 24 h post-final inhalation as shown in Fig. 6. GHS significantly suppressed the increase of IL-4 production induced by OVA treatment. However, there was no significant increase at 1 h post-final inhalation for any stimulation time examined (data not shown).

Effect of GHS on IL-4 Production by OVA at 24 h Post-Inhalation

BALB/c mice can develop an IL-4-dependent asthma. In the present study, a significant increase of IL-4 production was obtained when splenocytes were stimulated with OVA for 72 h at 24 h post-final inhalation as shown in Fig. 6. GHS significantly suppressed the increase of IL-4 production induced by OVA treatment. However, there was no significant increase at 1 h post-final inhalation for any stimulation time examined (data not shown). These results indicate that GHS may inhibit asthma development via up-regulation of IFN-γ production.

DISCUSSION

Experimental mouse models of allergic asthma established almost 10 years ago have provided new opportunities to study the disease pathogenesis and to develop new therapeutics. Though mouse models do not always reproduce all the features of human disease, wild-type mice develop a clinical syndrome that closely resembles allergic asthma, characterized by eosinophilic lung inflammation, AHR, increased IgE, mucus hypersecretion, and eventually, airway remodeling. Although the mouse models are not exact replicas of the human disease, observations in these models of allergic asthma support many existing paradigms.

It has been demonstrated that the Th1/Th2 cell response is shifted to a predominantly Th1 cell response during autoimmune diseases, while an overwhelming Th2 response elicits allergic disorders. Investigations on the balance of Th1/Th2 cytokines production should be helpful to understand the outcomes of different immune responses, and are clinically useful in treating immunologically dysregulated states.

GHS, originally described in the book “Experiences of
Zhu Danxi” (written by Zhu Danxi, a famous doctor of the Yuan Dynasty in China), has been clinically prescribed for hundreds of years to treat respiratory tract diseases such as winter colds, respiratory infection, allergic rhinitis, and bronchial asthma. Our previous studies showed that GHS could significantly enhance the survival times of Weiqi immunodeficient mice; it also enhanced the cold-adaptability of immunologically defective mice.29 We also demonstrated that GHS had preventive and curative effects on acute bacterial rhinitis, and could alleviate the inflammatory reaction and tissue injury induced by acute bacterial inflammation (data not shown; to be published elsewhere).

In this study, we demonstrated that GHS down-regulates the over-production of IgE and IL-4 via a significant and persistent increase of IFN-γ. Yang et al.30 found that Astragalus Root can significantly promote IFN-γ, IgG1, IgG2 and IgG3 production from peripheral blood monocyte (PBMC) in vitro, but had no significant effect on the production of IL-4 or IgG4, suggesting that it can modulate Th1/Th2 function and enhance the immuno-function of asthma sufferers by improving Th1-deficiency. GHS is commonly used to treat respiratory diseases in clinical practice in oriental countries, and there is currently much interest in the mechanisms of its effects on the immune system. An understanding of these may provide a pharmacological basis for more effective clinical application of GHS.

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