

LETTER TO THE EDITOR

IN VITRO ACTION OF SHO-SEIRYU-TO ON ALLERGEN-EXPOSED MONONUCLEAR CELLSM. TANAKA¹, K. INOUE¹, Y. KITAMURA¹, A. SHIMADA² and H. TAKANO³

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Although Sho-seiryu-to (SST), used as a traditional herbal (Kampo) medicine mainly in China and Korea, is shown to have immunomodulating potential, such as an anti-allergic one, its underlying mechanism has not been completely clarified. To partially address the issue, we explored its effects on allergen-exposed mononuclear cells. Male balb/c mice were intraperitoneally administered ovalbumin (OVA: 20 µg) plus alum or vehicle twice (Day 0 and Day 14). At Day 21, mice were sacrificed and splenocytes (mononuclear cells) were isolated and cultured in the presence or absence of OVA with or without SST. Thereafter, helper T-related cytokines in the culture supernatants were evaluated by means of ELISA. Protein level of interferon-γ was lower than 5.0 pg/mL in the supernatants from OVA-non-exposed or -exposed mononuclear cells in the presence or absence of OVA stimulation. On the other hand, SST induced the cytokine from both types of mononuclear cells in the presence ($P < 0.05$) or absence of OVA stimulation as compared to corresponding control. By contrast, interleukin (IL)-4 level tended to be decreased by SST in OVA-non-exposed mononuclear cells as did IL-13 in both non-exposed and exposed mononuclear cells as compared to vehicle. In conclusion, immunoregulating efficacy by SST on allergy-prone subjects may include, at least in part, restoring helper T balance mainly through hyperproduction of IFN-γ against mononuclear cells such as lymphocytes.

The prevalence of allergic disorders has rapidly increased, especially in urban areas, over the past decades (1). Although it has not been directly correlated with a fatal outcome, the irritable symptoms reduce labor effectiveness and the quality of life of patients and the economic burden of this condition accounts for some rates (2, 3). Mechanistically, T helper 2 response and consequent IgE-mediated pathobiology in mast cells/basophils could be largely involved in the development of allergic diseases, therefore, its treatment has generally

focused on suppressing these cellular and molecular processes, which represent antihistamines, steroids, and thromboxane A₂ and leukotriene receptor antagonists. However, long-term use of these drugs can evoke adverse health reactions such as weight gain and liver damage, as well as antihistamine- and steroid-related reactions; thus, alternative therapeutic approach strategy, possibly in combination with these previous treatments, is required to minimize these reactions.

A herbal (Kampo) medicine, Sho-seiryu-to (SST),

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has long been prescribed for supportive/complement therapy against allergic diseases as well as stressful pathologic conditions such as inflammation, cough, and pain etc. Its pharmaceutical efficacy is shown in clinical *in vivo*, and *in vitro* studies (4, 5). Nonetheless, its underlying mechanism regarding immunomodulating potential, including the anti-allergic one, has not been fully clarified, particularly in terms of its targeting points in allergic immunological processes.

The aim of the present study was to explore its *in vitro* action on allergen-exposed mononuclear cells by means of an *ex vivo* assay.

MATERIALS AND METHODS

Animals

Seven-week-old balb/c male mice weighing 24-27 g were purchased from SLC Japan Inc., (Shizuoka, Japan) and were given sterile distilled water and a commercial diet (Labo MR Stock; Nosan Corporation Life-Tech Department., Yokohama, Japan) *ad libitum*. They were housed under conventional conditions in an animal facility that was maintained at 24-26°C with 55-75% humidity and a 12-h light/dark cycle. All animal studies were approved by the Institutional Review Board of International University of Health and Welfare.

Reagent

The SST preparation used was a dry brown powder of the aqueous extract of Sho-seiryu-to (Tsumura & Co., Tokyo, Japan). SST (0.1 g/mL) was suspended in a phosphate-buffered saline (PBS, Gibco-Invitrogen, Carlsbad, CA, USA).

Preparation of mononuclear cells (spleen cells)

Mice were intraperitoneally administered ovalbumin (OVA: 20 µg/mouse) plus alum or vehicle twice (Day 0 and Day 14). At Day 21, mice were anesthetized with sodium pentobarbital (Dainippon Pharmaceutical Co., Osaka, Japan) given intraperitoneally (50 mg/kg), and the spleens were removed. After removal, the spleens were pushed through a nylon mesh and red blood cells were lysed with ammonium chloride. The cells were centrifuged at 400'g for 5 min at 20°C. After washing with PBS, the cells were resuspended with AUM V medium (Gibco-Invitrogen). The numbers of viable cells were determined by the trypan blue (Invitrogen, CA, USA) exclusion method.

Measurement of cytokine production by mononuclear cells (spleen cells)

Levels of interferon (IFN)-γ and interleukin (IL)-4,

IL-5, and IL-13 produced by mononuclear cells (spleen cells) were measured as previously described with slight modification (6). The mononuclear cells (5×10^6 cells/mL) from normal or antigen-immunized mice were suspended in an AIM V culture medium (Gibco-Invitrogen) and then cultured in the presence or absence of OVA (1 mg/mL) with or without SST for 72 h at 37°C in a CO₂ incubator. IFN-γ (Thermo Fisher Scientific Inc., Waltham, MA, USA), IL-4 (R&D Systems Inc., Minneapolis, MN, USA), IL-5 (Thermo Fisher Scientific Inc.), and IL-13 (R&D Systems Inc.) were measured using each ELISA kit according to the manufacturer's instructions (n = 5 in each group).

Statistical analysis

Data was expressed as mean ± standard deviation (SD). The Mann-Whitney U test was used to determine differences between groups for all data obtained in this study. The *p*-value < 0.05 was considered statistically significant.

RESULTS

Protein level of IFN-γ was lower than 5.0 pg/mL in the supernatants from OVA-non-exposed or -exposed mononuclear cells in the presence or absence of OVA stimulation. On the other hand, SST significantly induced the cytokine from either types of mononuclear cells in the presence (*P* < 0.05) or absence of OVA stimulation as compared with corresponding control (Table I). By contrast, IL-4 level tended to be lower in the supernatants from OVA-non-exposed mononuclear cells incubated with SST than those from cells incubated with vehicle in the presence or absence of OVA rechallenge (Table I). IL-13 level also tended to be lower in the culture supernatants from both OVA-non-exposed and -exposed mononuclear cells treated by SST than those from cells by vehicle in the presence or absence of OVA (Table I).

DISCUSSION

From a scientific point of view, anti-inflammatory effects of SST have been shown in humans assessed in a double-blind randomized study on patients with allergies such as bronchial asthma and allergic rhinitis (7). Furthermore, there are several *in vivo* reports concerning SST immunomodulatory action on allergic pathology, such as suppression of Th2

Table I. Effects of *Sho-seiryu-to* (SST) on interferon- γ , interleukin (IL)-4, and IL-13 production/release from mononuclear cells with or without antigen exposure.

OVA-nonexposed mouse				OVA-exposed mouse			
group	IFN- γ (pg/mL)	IL-4 (pg/mL)	IL-13 (pg/mL)	group	IFN- γ (pg/mL)	IL-4 (pg/mL)	IL-13 (pg/mL)
Control	1.32 $\pm$ 1.61	3.42 $\pm$ 0.86	8.95 $\pm$ 3.75	Control	0.06 $\pm$ 0.12	2.89 $\pm$ 0.38	6.36 $\pm$ 2.40
SST	6.59 $\pm$ 5.13	2.30 $\pm$ 1.39	5.37 $\pm$ 2.96	SST	6.88 $\pm$ 12.03	2.89 $\pm$ 0.82	4.55 $\pm$ 0.89
OVA	1.89 $\pm$ 3.78	3.38 $\pm$ 2.19	6.51 $\pm$ 4.28	OVA	0.00 $\pm$ 0.00	2.46 $\pm$ 2.04	6.62 $\pm$ 2.27
SST + OVA	12.40 $\pm$ 8.43 **	1.55 $\pm$ 1.18	4.91 $\pm$ 3.52	SST + OVA	3.06 $\pm$ 2.02 **	2.90 $\pm$ 1.20	5.06 $\pm$ 1.92

The levels of IFN- γ , IL-4, and IL-13 in the mononuclear cell culture supernatant after treatment with SST for 72 h were measured by ELISA. Data shown are mean $\pm$ SD of five animals per group. * p < 0.05 vs control, ** p < 0.05 vs OVA.

differentiation at the level of synapse between antigen-presenting cells and CD4⁺ T cells (7), inhibition of histamine signaling targeting its receptor expression and synthesizing enzyme (8), and augmentation of NGF induction accompanied by allergic pathology. On the other hand, mechanistic studies, including those *in vitro*, are fewer.

In the present study, we focused on *in vitro* action of SST on mononuclear cells (spleen cells), since these cell compartments, especially antigen-sensitized ones, are crucial for Th2-mediated immunological pathophysiology and pathobiology such as allergic diseases. Ikeda et al. previously examined *in vivo* effects of SST on OVA recall response and showed that splenic mononuclear cells isolated from SST-administered mice produced less IL-4 following *in vitro* OVA stimulation as compared to those from control mice, whereas there was no difference in levels of IFN- γ between these experimental groups (7). That study suggests that *in vivo* pretreatment with SST attenuates antigen-related recall response likely through suppression of Th2 polarization. We therefore examined effects of SST on *in vivo* allergen-exposed mononuclear cells by measuring Th cytokines produced by allergen stimulation in the presence of SST. As a result, SST induced IFN- γ in the presence or absence of the stimulation as compared to each control from both allergen-non-exposed and -exposed mononuclear cells, in contrast, it tended to suppress IL-4 level from allergen non-exposed mononuclear cells and IL-13 level from both non-exposed and exposed ones without statistical significance, suggesting that immunoregulating efficacy by SST on allergy-prone subjects may include, at least in part, restoring of

helper T balance mainly through hyperproduction of IFN- γ against these cells. In other words, SST can provide different immunoregulatory action on different allergy-related cascade/immune cell components, leading to achievement of anti-allergic potential. Chang et al. have shown that SST serves as anti-viral infection *in vitro* (4). Hot water extract of SST dose-dependently reduced viral load of human respiratory syncytial virus infected in both human upper and lower respiratory tract cells. In their study, SST extract can stimulate these respiratory tract cells to induce IFN- γ , implying its underlying machinery of anti-viral effects. Therefore, our study may add alternative biological evidence that SST can also stimulate hematopoietic cells to induce IFN- γ under allergic or non-allergic milieu.

Limitation involves the matter that the current study failed to examine *in vivo* pharmaceutical effects on OVA-sensitized mononuclear cells. Although, as mentioned above, Ikeda et al. have previously elucidated the effects, their experiments did not cover direct effects on these cells (7). Furthermore, to investigate the impact of SST on helper T balance, for example, sorting CD4⁺ cells by means of flow cytometry may be strictly needed. Similarly, as *in vitro* analyses, we should use metabolites of SST to reproduce the phenomenon in the body. Furthermore, success in exploring responsible constitutive substances (alternatively, combination of ingredients) among SST for anti-allergic efficacy will bring a beneficial progress in both Kampo medicine and allergology. However, at this time, it is quite difficult to examine these experiments using metabolites or some portions, since SST is constituted mainly by 8 kinds of "shoyaku". Finally, in the current study, these

mononuclear cells from mice repeatedly exposed to allergen (OVA) did not seem to be sufficiently sensitized by the allergen, since *in vitro* allergen rechallenge did not significantly activate production/release of Th2 cytokines. However, we confirmed that serum value of OVA-specific IgG₁, an important antibody for allergic pathology in mice, was significantly ($P < 0.01$) higher in OVA-exposed mice than in non-exposed ones, indicating that systemic immunization was established on the current *in vivo* protocol (OVA-non-exposed vs -exposed; 1.1 ± 0.1 vs 94.1 ± 34.1 [U/mL]; $n = 9$ in each group). It is likely that the almost negative results of Th2 cytokine profiles are attributable to the *in vitro* one, such as cell number (per well), OVA dosage, and/or endpoint (incubation duration); thus we will elucidate these points to validate the action of SST. We will also consider further examination using other allergens including house dust mite, cockroach, mould, etc. on other strains of mice such as C57BL/6, C3H/He, and NC/Nga. Nonetheless, the current cutting edge study achieved optimal results consistent with previous ones in terms of regulation of helper T response by SST, and provides a scientific hint for future research to certainly prescribe this herbal medicine as supportive strategy for patients with immunodysregulated conditions such as allergy.

In conclusion, SST induced IFN- γ in the presence or absence of antigen stimulation as compared to control from antigen-exposed or -non-exposed splenic mononuclear cells. In contrast, IL-4 level tended to be decreased by SST in OVA-non-exposed mononuclear cells and IL-13 was in both non-exposed and exposed mononuclear cells as compared to vehicle. Immunomodulatory action of SST in allergic subjects, previously suggested by several researchers, might be, at least in part, attributable to restoring helper T balance mainly through hyperproduction of IFN- γ against these cells, such as lymphocytes. The current study provides a suggestion for complementary therapeutic possibilities for allergy and related illness.

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