Effect of Sairei-to (Chai-Ling-Tang) on adjuvant-induced arthritis in Lewis rats

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Abstract

We tested the effects of oral treatment with Sairei-to (Chai-Ling-Tang, 柴苓湯) on adjuvant-induced arthritis (AA) of rats, an experimental model of rheumatoid arthritis. Sairei-to significantly suppressed paw swelling in a systemic phase, but did not in an acute phase by daily oral administrations from the day of adjuvant inoculation. Unlike glucocorticoids, Sairei-to did not induce significant changes in the weights of the body, thymus, adrenal gland, spleen or lymph nodes, or in the percentages of CD3- and CD4-positive cells in the spleen and lymph nodes. However, Sairei-to inhibited the spontaneous proliferation of spleen cells and lymph node cells in AA rats, and suppressed the proliferation of the spleen cells and the IFN-γ production in the lymph nodes in response to heat-killed *Mycobacterium butyricum* (*M. butyricum*) significantly. Additionally, the administration of Sairei-to from the 16th day after the adjuvant inoculation (when the paw swelling reached a maximum point in the second inflammation), clearly suppressed the paw swelling. These results suggested that Sairei-to improved AA via an immunosuppressive modulation of arthritic T cell function.

Key words Sairei-to, adjuvant arthritis, T cell, proliferative response, interferon-γ.

Abbreviations AA, adjuvant arthritis ; CFA, complete Freund's adjuvant ; ELISA, enzyme-linked immunosorbent assay ; FITC, fluorescein-isothiocyanate ; IFN-γ, interferon-γ ; *M. butyricum, Mycobacterium butyricum* ; PE, phycoerythrin ; PMS, 1-methoxy-5-methylphenazinium methylsulfate ; RA, rheumatoid arthritis ; Sairei-to (Chai-Ling-Tang), 柴苓湯 ; WST-1, 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H-tetrazolium, monosodium salt.

Introduction

Adjuvant-induced arthritis (AA) in rats has been widely used as a model of rheumatoid arthritis (RA), which is a chronic disease accompanied with pannus formation, durability of synovium, and damage to cartilage and bone erosion, giving rise to the destruction of joints.[1,2] Previous studies have indicated that T lymphocytes, especially CD4 cells, play an important role in the pathogenesis of AA and RA.[3,4] For example, the inoculation of an arthritic T cell clone established in AA rats to irradiated normal rats induced arthritis,[5,6] and anti-CD4 antibody can suppress both AA in rats and human RA.[7,8] Interferon (IFN)-γ appears to accelerate the progression of AA, at least at the stage of induction, because treatment with anti-IFN-γ antibody at this stage suppresses the AA progression.[9,10] However, the pathogenesis of AA and RA have not yet been conclusively elucidated. Thus, various medicines such as glucocorticoids, non-steroidal drugs (NSAID) and disease-modifying anti-rheumatic drugs (DMARD) with immunosuppressive, anti-inflammatory or immunomodulatory effects cannot cure RA completely and induce many kinds of side effects after long-term use.

Many Kampo medicines have been used for the treatment of various chronic diseases. Sairei-to (柴苓

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湯), a Kampo medicine, has been used for the treatments of RA,[11,12] nephrosis syndrome[13] and hepatitis,[14] mainly in combination with glucocorticoids. The use of Sairei-to allows the reduction of the dose of glucocorticoids and ameliorates its side effects.[11,14] Although Sairei-to has been shown to induce endogenous glucocorticoids,[15] the mechanism underlying this effect is not yet known. Sairei-to itself has been reported to have immunomodulatory and anti-inflammatory effects.[16] In AA rats, Sairei-to and other Kampo medicines have been shown to inhibit paw swelling.[17,18]

In this study, we examined the inhibition of rat AA by the oral administration of Sairei-to, and its underlying mechanism.

Materials and Methods

Induction of AA : Female Lewis rats were purchased from Charles River Japan Inc. (Yokohama, Japan). Rats (8 weeks of age) were injected intradermally into a right hindpaw with 0.1 ml of complete Freund's adjuvant (CFA) containing 5 mg of heat-killed *Mycobacterium butyricum* (*M. butyricum*, Difco Laboratories, Detroit, MI) in 1 ml of liquid paraffin (Wako, Osaka).

The development of polyarthritis was assessed by measuring the injected (right) and uninjected (left) paw volume with an electronic plethysmometer (MK-550, Muromachi Kikai Co. Ltd, Tokyo, Japan).

Sairei-to : Sairei-to was prepared from a mixture of 12 medical herbs in the following proportion : Bupleuri Radix (7.0 g), Pinelliae Tuber (5.0 g), Scutellariae Radix (3.0 g), Zizyphi Fructus (3.0 g), Glycyrrhizae Radix (2.0 g), Zingiberis Rhizoma (4.0 g), Ginseng Radix (3.0 g), Cinnamomi Cortex (2.0 g), Atractylodis Rhizoma (3.0 g), Alismatis Rhizoma (5.0 g), Poluporus (3.0 g), and Hoelen (3.0 g). Extraction was carried out by refluxing one-part herb mixture with ten-parts water at 95-100°C for 1 hour. The extract was then spray-dried in a hot air stream (recovery : about 15 %).

Sairei-to was suspended in distilled water, and rats were orally administered 250 mg/kg of Sairei-to daily from day 0 or day 16 after the adjuvant injection. Control rats were orally administered an equal volume of distilled water.

Flow cytometry analysis : Spleen cells or iliac lymph node cells were suspended in AUTO-POW Eagle MEM (ICN, Costa Mesa, CA) supplemented with 2 % fetal calf serum (Hyclone Lab., Logan, UT) and stained with fluorescein-isothiocyanate (FITC)-conjugated anti-rat CD3 monoclonal antibody (CALTAG, So. San Francisco, CA) or phycoerythrin (PE)-conjugated anti-rat CD4 monoclonal antibody (CALTAG), and then washed twice with MEM. Fluorescence-positive cells were analyzed by a FACScan (Becton Deckinson, San Jose, CA).

The percentage of positive cells was calculated in the gated lymphocytes for spleen and iliac lymph node cells.

Proliferation of adjuvant-sensitized lymph node or spleen cells in response to M. butyricum : Lymph nodes (iliac and inguinal), spleen, adrenal, or thymus were obtained from normal rats and AA rats on day 17 after the adjuvant inoculation. After measurement of these weights, the lymph node cells or spleen cells were suspended in RPMI 1640 (Nissui Seiyaku Co., Tokyo) medium containing 10 % heat inactivated fetal calf serum, supplemented with 10 mg/ml gentamicin sulfate (Schering-Plough, Osaka).

The lymph node cells were cultured with 1 or 10 μg/ml of heat-killed *M. butyricum* or without for 72 hours at the cell density of 5×10^5/well in a 96-well culture plate. The spleen cells were cultured with 0.1 or 1 μg/ml or without for 72 hours at the cell density of 4×10^5/well. After the incubation, the proliferative responses of the cells were assessed by addition of 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-1) solution (1 mM WST-1 and 0.2 mM 1-methoxy-5-methylphenazinium methylsulfate (PMS)) (DOJINDO, Kumamoto, Japan) and followed by reading of the absorbance at 450 nm with 630 nm as a reference wavelength by using a microplate reader.

IFN-γ production by adjuvant-sensitized lymph node and spleen cells in response to M. butyricum : Iliac and inguinal lymph nodes or spleen were obtained from normal rats and AA rats on day 17. The mixed lymph node or spleen cells from 4 rats in a group were suspended in RPMI 1640 medium. The lymph node cells were cultured with 1 or 10 μg/ml

heat-killed *M.butyricum* for 72 hours at the cell density of 5×10^6/well in a 24-well culture plate. The spleen cells were cultured with 0.1 or 1 μg/ml for 48 hours at the cell density of 4×10^6/well. After the incubation, the culture supernatants were collected for the determination of IFN-γ.

The concentration of IFN-γ was determined with the use of an enzyme-linked immunosorbent assay (ELISA) kit (Hycult Biotechnology, Netherlands).

Results

Effects of Sairei-to on paw swelling in adjuvant-induced arthritic rats

Adjuvant arthritis is characterized by two stages ; an acute phase manifesting the swelling of the CFA-injected paw and a later systemic phase characterized by paw swelling of both feet. The daily oral administration of Sairei-to (250 mg/kg) beginning on the day of the adjuvant injection (day 0) significantly inhibited the swelling of both paws measured on day 16, at the systemic phase (Fig. 1-B). Similar results were

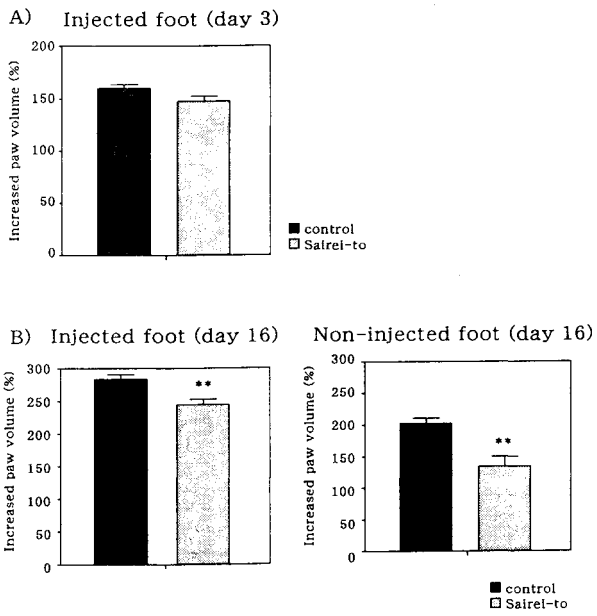

Fig. 1 Effect of Sairei-to on the induction of AA.
Lewis rats were orally administered Sairei-to (250 mg/kg) or water (control) daily from day 0 to day 16. The values of paw swelling were expressed as means±S.E. of the percent measure in the volume compared with that before adjuvant injection. n=8 (control), n=7 (Sairei-to)
**; $p<0.01$ vs. control (Student's t-test)

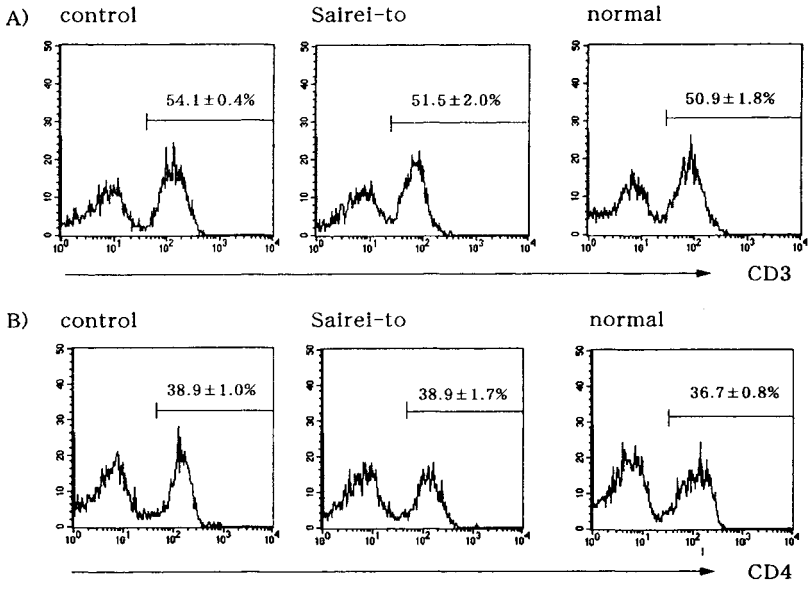

Fig. 2 Effect of Sairei-to on spleen lymphocyte subsets in Lewis rats. Rats were orally administered Sairei-to (250 mg/kg) or water (control) daily from day 0 to day 16. The spleen cells were obtained on day 17 after adjuvant injection. The lymphocyte-gated spleen cells were analyzed by a flow-cytometry. The values were expressed as means±S.D. n=5 (control, normal) n=4 (Sairei-to)
A) anti-CD3 antibody stain, B) anti-CD4 antibody stain

obtained on day 14 (data not shown). Sairei-to showed no suppressive effect on the paw swelling on day 3 (the acute phase) (Fig. 1-A).

Flow cytometry analysis of rat spleen cells and lymph node cells

For the examination of the effects of Sairei-to on lymphocyte subsets, spleen cells obtained from AA rats on day 17 were stained with fluorescence-labeled anti-CD3 or anti-CD4 antibody, and analyzed by flow-cytometry. The percentages of CD3- and CD4- positive cells did not differ significantly between the normal and arthritic rats, or between the arthritic rats with and without Sairei-to treatment (Fig. 2). Similar results were obtained in the iliac lymph node cells.

The adjuvant injection induced increases in the weights of the spleens and lymph nodes, but Sairei-to had no effect on these changes (data not shown). Additionally, Sairei-to did not induce changes in the weights of the body, thymus and adrenal gland (data not shown).

Mycobacterial antigen-specific proliferative response of lymph node cells and spleen cells

The effects of Sairei-to on the proliferative response of spleen cells and lymph node cells to *M. butyricum* were examined in AA rats on day 17.

Spontaneous proliferations of spleen cells and lymph node cells without *M.butiricum* was observed in AA control rats (Fig. 3). The spontaneous responses were significantly suppressed by Sairei-to treatment. Dose-dependent proliferative responses of lymph node cells from AA rats to *M.butyricum* were observed, whereas in cells from normal (non-treated) rats, such responses were scarcely observed (Fig.3-A). Sairei-to

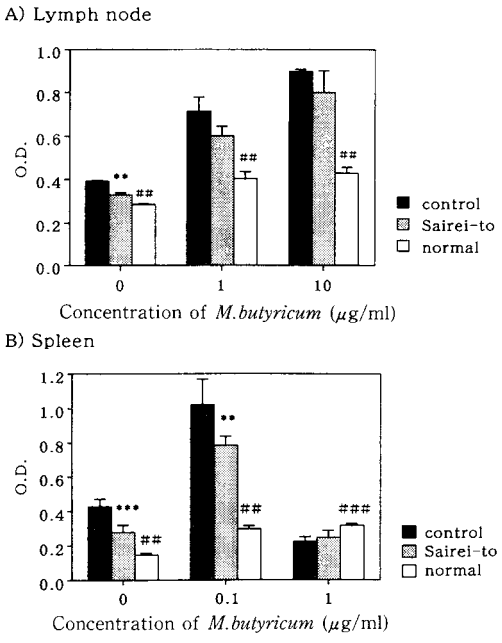

Fig. 3 Effect of Sairei-to on proliferative response of lymph node cells (A) and spleen cells (B) to *M.butyricum* in AA rats.
Rats were orally administered with Sairei-to (250 mg/kg) or water (control) daily from day 0 to day 16. On day 17 after adjuvant injection, these cells were obtained from 4 rats per group and pooled in each group. These cells were triplicately (A) or quintuplicately (B) cultured with *M. butyricum* for 72 hr. The values were expressed as means±S.D.
*** and ###; $p<0.001$ vs. control, ** and ##: $p<0.01$ vs. control (Student's t-test)

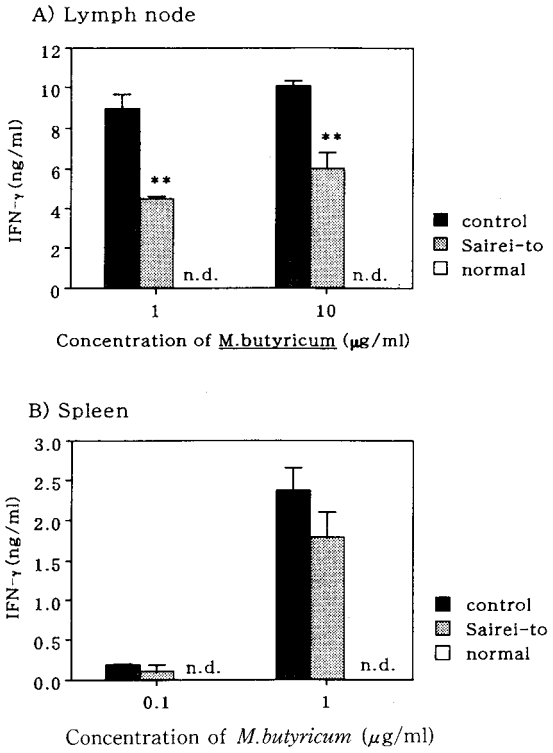

Fig. 4 Effects of Sairei-to on IFN-γ production of lymph node cells (A) and spleen cells (B) stimulated with *M. butyricum* in AA rats. Rats were orally administered Sairei-to (250 mg/kg) or water (control) daily from day 0 to day 16. On day 17 after adjuvant injection, these cells were obtained from 4 rats per group and pooled in each group. These cells were triplicately cultured with *M.butyricum* for 48 hr. n.d.=not detected. IFN-γ was not detected in the culture supernatants of the cells from the normal (non-treated) rats stimulated with *M.butyricum*. The values were expressed as means±S.D. ** ; $p<0.01$ vs. control (Student's t-test)

suppressed slightly the responses but not significantly. The AA control rat spleen cells proliferated in response to stimulation with 0.1 μg/ml of *M.butyricum*, but not in response to 1 μg/ml (Fig. 3-B). Sairei-to significantly suppressed the response.

IFN-γ production in the culture supernatants of lymph node cells and spleen cells stimulated with *M. butyricum*.

The IFN-γ production by spleen cells and lymph node cells stimulated with *M.butyricum* was tested. The IFN-γ production by the lymph node cells of AA control rats was significantly suppressed by Sairei-to treatment (Fig. 4-A) ; that in the spleen cells was slightly suppressed by Sairei-to treatment, but not significantly (Fig. 4-B).

Therapeutic effects of Sairei-to on paw swelling in AA rats

Sairei-to seemed to modify some immunological functions in the AA rats. Therefore, the therapeutic effect of Sairei-to on established AA was examined. Rats were orally administered Sairei-to daily from day 16 after adjuvant injection.

Sairei-to significantly suppressed the paw swelling in AA rats (Fig. 5).

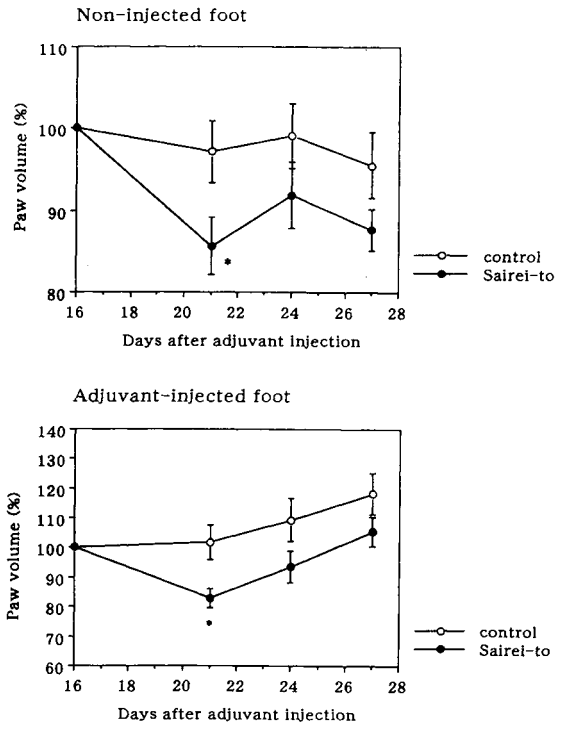

Fig. 5 Therapeutic effect of Sairei-to on adjuvant arthritis.
Lewis rats were orally administered with Sairei-to (250 mg/kg, n=12) or water (control, n=12) daily from day 16. The values of paw swelling were expressed as means± S.E. of the percent in the volume compared with the increased volume between day 0 and day 16.
*; $p<0.05$ vs. control (Student's t-test)

Discussion

In the acute phase, the peak of swelling in only an adjuvant injected foot was observed between day 2 and day 5. In the systemic phase, the swelling of both adjuvant injected and non-injected feet reached to the maximum level about day 14 ~ day 16. We estimated the paw swelling on day 3 in acute phase, and on day 16 or day 14 in the systemic phase. We observed that the daily treatment with Sairei-to starting on the day of adjuvant injection significantly suppressed the paw swelling of the adjuvant-injected foot and non-injected foot in the later systemic phase of AA, but not in the acute phase.

Sairei-to also showed a therapeutic effect on established AA by administration from day 16. Additionally, in AA rats and patients suffering from RA, anti-CD4 antibody therapy has been shown to result in an improvement of arthritis.[7,8] These observations suggest that CD4 T cells play an important role in the development of arthritis. However, no significant difference has been found between RA patients and healthy persons in the percentages of CD3, CD4, or CD8 T cells in peripheral blood.[19] Similarly to the previous results, we observed no significant differences in the percentages of CD3 and CD4 T cells in the spleen and lymph nodes between normal and AA rats. Sairei-to did not alter the percentages of CD3 or CD4 in the spleen and lymph nodes, or the weights of these organs. These results suggested that the inhibition of AA by Sairei-to is not due to the decrease of CD3 or CD4 positive T cell.

The estimation of cellular functions showed that Sairei-to inhibited the spontaneous proliferation, presumably autoreactive T cell response, of spleen cells and lymph node cells from AA rats, and the proliferation of the spleen cells in response to *M.butyricum* (Fig. 3). These results suggested that Sairei-to suppresses the development of T cells reactive to *M.*

butyricum and autoantigen in AA rats. Additionally, Sairei-to inhibited the IFN-γ production from the lymph node cells in response to *M.butyricum* (Fig. 4). IFN-γ has been reported to accelerate the progression of AA.[9,10] The allergic inflammation mediated by T cells which produce IFN-γ, is type IV. Sairei-to have been reported to have a tendency to inhibit a type IV allergic reaction.[20] Type IV allergic reactions inhibited by glucocorticoids, an immunosuppressive drug. In a previous study, Sairei-to has been shown to induce endogenous glucocorticoids.[15] Therefore, the present effect of Sairei-to may be caused by endogenous glucocorticoids. However, Sairei-to did not restrain acute inflammation in the present study, and the treatment with Sairei-to was not accompanied by such side effects as the reduction of body weight and atrophy of thymus, adrenals, spleen and lymph nodes which have been observed in glucocorticoid-treated experimental animals.[18,21] Sairei-to has been reported to regulate the production of some cytokines, including IFN-γ.[22,23] Here, Sairei-to treatment suppressed not only the induction of AA but also established AA, without an anti-acute inflammatory effect. These results suggest that Sairei-to improved AA via an immunosuppressive modulation of arthritic T cell function.

和文抄録

慢性リウマチ関節炎の実験モデルとされているアジュバント関節炎ラットに，柴苓湯を経口投与し，その効果を検討した。柴苓湯をアジュバント投与日から投与した場合，一次炎症には効果を示さなかったが，二次炎症における足浮腫を有意に抑制した。グルココルチコイドと異なり，柴苓湯の投与による体重の減少や胸腺，副腎，脾臓，リンパ節の重量の減少，そして脾臓およびリンパ節細胞のCD3及びCD4の発現比率の変化も認められなかった。柴苓湯はアジュバント関節炎における，脾臓およびリンパ節細胞の自己増殖を抑制し，結核死菌刺激による脾臓細胞の増殖反応及びリンパ節細胞のIFN-γの産生を有意に抑制した。更に，二次炎症の発症がピークに達するday 16より柴苓湯を投与した場合においても，足浮腫を有意に抑制した。

これらの結果より，柴苓湯は，アジュバント関節炎を誘導するT細胞の機能を抑制することにより，足浮腫を抑制することが示唆された。

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